REMARKS

Reconsideration and withdrawal of the rejections of the application are respectfully requested in view of the amendments, remarks, and enclosures herewith.

I. STATUS OF THE CLAIMS AND FORMAL MATTERS

Claims 1, 4-15, and 17-78 are now pending. Claim 1 has been amended herein, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents.

No new matter is added.

It is submitted that these claims are in full compliance with the requirements of 35 U.S.C. §112. The amendments to the claims and the remarks herein are not made for the purpose of patentability within the meaning of 35 U.S.C. §§ 101, 102, 103 or 112; but rather the amendments and remarks are made simply for clarification and to round out the scope of protection to which Applicants are entitled. Support for the amended claims can be found throughout the specification, and specifically at paragraph 36 of the specification as published (US 2004/0204352).

II. CLAIM OF PRIORITY

The Office Action indicated that a certified copy of UK 0209884.6 had not been received, such that the benefit of the foreign priority filing date was not granted. Applicants respectfully submit that on June 16, 2008, a certified copy of UK 0209884.6 was submitted to the Patent and Trademark Office. Thus, Applicants respectfully submit that the requirements for claiming priority have been satisfied, and grant of the claim of priority by the Office is respectfully requested.

III. THE DOUBLE PATENTING REJECTIONS ARE OVERCOME

Claims 1, 15 and 78 were rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 30, 31 and 39 of co-pending U.S. Patent Application No. 10/579,113. The rejection is respectfully traversed.

Applicants respectfully submit that co-pending U.S. Patent Application No. 10/579,113 has not yet been examined. Accordingly, the double patenting rejection should be provisional

only, as it is impossible to predict whether the claims that would eventually be granted in both the present application and USSN 10/579,113 would be subject to a double patenting rejection. Thus, Applicants request that the double patenting rejection be withdrawn in this application, to be issued later in co-pending 10/579,113 following prosecution, if warranted by the claims allowed in each application.

Thus, reconsideration and withdrawal of the double patenting rejection is respectfully requested.

IV. THE REJECTIONS UNDER 35 U.S.C. §112 2nd PARAGRAPH ARE OVERCOME

Claims 1, 10-15, 20, 21 and 78 were rejected under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. The rejection is respectfully traversed.

The rejection is based on the recitation of "an isolated polypeptide comprising an extracellular domain as recited in SEQ ID NO :22." The Office Action alleges that it is unclear "if applicant intends the extracellular domain to comprise or consist of SEQ ID NO: 22 or some unspecified part thereof." Claim 1 has been amended herein to specifically indicate that the isolated polypeptide comprises or consists of i), ii), or iii), and that the polypeptide additionally comprises an extracellular domain as recited in SEQ ID NO: 22. Further, the recitation of SEQ ID NO: 22 has been omitted from part ii). Thus, Applicants respectfully submit that the claims are now clear that the polypeptide additionally comprises an extracellular domain as recited in SEQ ID NO: 22 in addition to one of i), ii), or iii), such that the claim is now definite.

Claim 13 was rejected for reciting "a compound that either increases or decreases the level of expression or activity of a polypeptide…without inducing any of the biological effects of the polypeptide."

Applicants respectfully submit that this phrase is not indefinite. Rather, as described in the specification as filed at page 41, lines 23-28, the claim describes the situation wherein "binding of the polypeptide to normal cellular binding molecules may be inhibited, such that the normal biological activity of the polypeptide is prevented". Thus, when read in view of the specification, the claim is definite.

Consequently, reconsideration and withdrawal of the rejections under 35 U.S.C. §112, second paragraph, are respectfully requested.

V. THE REJECTIONS UNDER 35 U.S.C. §112 1st PARAGRAPH ARE OVERCOME

Claims 1, 10-15, 20, 21 and 78 were rejected under 35 U.S.C. §112, first paragraph, because the specification allegedly lacks enablement for all of the claimed elements. The rejection is respectfully traversed.

The Office Action admits that the specification is enabled for the full length polypeptides of SEQ ID NOs: 16, 20, 22 and 26, or a fusion protein comprising a polypeptide which comprises or consists of the amino acid sequence of SEQ ID NOs: 16, 20, 22 or 26 fused to a heterologous polypeptide that has an activity that is an antagonist of TNF-alpha, IL-4, IL-6 or IL-2. However, the Office Action alleges that the specification does not provide enablement for the full scope of the claims.

Claims 15, 20 and 21 were also rejected under 35 U.S.C. §112, first paragraph, because the specification allegedly lacks enablement for all of the claimed elements. This rejection is also respectfully traversed. The Office Action indicates that the specification allegedly does not reasonably provide enablement for a polypeptide or fusion protein comprising a full length polypeptide for use in therapy and diagnosis of an inflammatory disease, an autoimmune disease, any generic liver disease or liver failure.

35 U.S.C. §112, first paragraph, requires that the specification describe how to make and use the invention. 35 U.S.C. §112, first paragraph, recites, in pertinent part:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same[.]

A patent claim is invalid if it is not, *inter alia*, supported by an enabling disclosure. The test for enablement requires a determination of whether any person skilled in the art can make and use the invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 8 U.S.P.Q.2d 1400, (Fed. Cir. 1988). The factors involved in determining whether there is sufficient evidence to support a finding of enablement include, among others, (1) the breadth of the claims, (2) the nature of the invention, (3) the state of the prior art, (4) the level of one of ordinary skill, (5) the level of predictability in the art, (6) the amount of direction provided by the

inventor, (7) the existence of working examples, and (8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *See Wands*, 858 F.2d at 737, 8 U.S.P.Q.2d at 1404.

Applying the law to the instant facts, all of the pending claims are enabled. As previously stated, the specification does provide guidance as to how to make and use fragments that retain the cytokine antagonist function of the full-length polypeptide and fragments of homologues that retain this function. Specifically, Applicants respectfully direct the Examiner's attention to Exhibit 1, attached, which provides additional data furnished by the Applicants that demonstrates the necessity of the recited extracellular domain of SEQ ID NO: 22 in the claimed polypeptides.

Furthermore, the pending claims have been amended herein such that claim 1 specifically requires that the isolated polypeptide comprises or consists of i), ii), or iii), and that the polypeptide additionally comprises an extracellular domain as recited in SEQ ID NO: 22. Thus, the Office Action's rebuttal of Applicant's prior arguments on the basis that claim 1 is indefinite is now moot.

In addition, Exhibit 1 relates to experiments carried out using the extracellular domain of INSP052, the INSP052EC protein, referred to in the present application as SEQ ID NO: 22, the cloning and expression of which is described in examples 2 and 3 of the pending application. This region is found in the INSP052 protein (SEQ ID NO: 16), and so the full length protein would share the activity identified for the active fragment upon which the experiments are based, as would other fragments containing the extracellular domain.

Example 1 from Exhibit 1 demonstrates how INSP052 modulates cytokine expression in a live mouse model. Such mouse models involving LPS-induced cytokine release are frequently used as a model for fulminant hepatitis treatment. INSP052EC is shown to decrease expression of IL-6 and TNFa. This indicates that INSP052EC could be used to treat fulminant hepatitis.

In Example 2 of Exhibit 1, INSP052EC is shown to reduce ear swelling in a model of contact hypersensitivity. This demonstrates that INSP052 is useful in treating T cell-mediated inflammation of the skin, such as found in contact dermatitis and psoriasis.

Therefore, the experimental evidence provided in Exhibit 1 shows that INSP052 is useful in treating various autoimmune/inflammatory disorders, confirming the description present in the specification regarding the usefulness of such polypetides in treating these disorders.

INSP052 is also identical to a protein described in the literature as Hepatocyte cell adhesion molecule (hepaCAM). Post-published documents such as Chung et al. (attached) and Moh et al. (attached) provide further evidence of the role of INSP052 in wound healing. Chung et al. discloses that hepaCAM increases cell spreading on the matrices fibronectin and matrigel as well as delaying cell detachment and enhancing wound healing in an in vitro wound healing assay, while Moh et al. shows that hepaCAM encodes an Ig-like transmembrane glycoprotein and is involved in cell adhesion and growth control. Both of these references further confirms Applicants description of the utility of the INSP052 protein.

Thus, when the confirmatory data attached as Exhibit 1 and the enclosed references are considered, in view of the fact that the techniques used in the present application allow highly accurate predictions of protein function to be made, the claims are clearly enabled.

To this end, the Examiner is again reminded of the text in the specification which teaches the skilled person to identify fragments that contain an immunoglobulin domain (page 8 of the application as filed) and the functional importance of this domain (page 16, line 4-9 of the application as filed). The extracellular domain of the INSP052 polypeptide contains an immunoglobulin domain and the data presented in the examples and in Exhibit 1 attached hereto, confirm that extracellular fragments retain the activity of the full-length polypeptides. The Examiner is further reminded that fragments consisting of the extracellular domain or of exons 2 and 3 of the INSP052 polypeptide are fully enabled by the specification since they are specifically disclosed on pages 10-11, in Example 2 and in Figure 6 of the application as filed. Again, such description of the polypeptide functions in concert with the attached data and references to demonstrate enablement.

Therefore, reconsideration and withdrawal of the rejections under 35 U.S.C. §112, first paragraph, are respectfully requested.

Claims 1, 10-15, 20 and 21 were rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the written description requirement. The rejection is respectfully traversed.

The Office Action indicates that the rejection is maintained because claim 1 is indefinite, such that "the claims "could reasonably be interpreted to recite fusion peptides comprising fragments of any one of SEQ ID NOs: 16, 20, 22 and 26. The claims do not require that the

polypeptide fragment thereof possess any disclosed distinguishing feature or specific activity." Office Action at 10.

Applicants respectfully submit that the claims have been amended herein to recite that the isolated polypeptide comprises or consists of i), ii), or iii), and that the polypeptide additionally comprises an extracellular domain as recited in SEQ ID NO: 22, and that the polypeptide functions as an antagonist of cytokine expression and/or secretion. Accordingly, even in those instances where a fragment of one of the recited sequences is present, the claimed isolated polypeptide must now at a minimum comprise an extracellular domain as recited in SEQ ID NO: 22 and have the required functionality.

As described above, the specification provides ample guidance as to those species which are encompassed by the currently pending claims. Specifically, the specification does provide detailed guidance as to how to make and use fragments that at a minimum comprise an extracellular domain as recited in SEQ ID NO: 22 and has the required functionality. The recited sequence, and the conservation of that minimum sequence in each of the claimed polypeptides thus provides an adequate description of the genus of claimed compounds.

Therefore, as the Applicants have adequately defined the claimed species, and provided the teaching needs to identify members of the species, the specification provides adequate written description. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

V. THE REJECTIONS UNDER 35 U.S.C. §102 ARE OVERCOME

Claims 1, 10-15, 20, 21 and 78 were rejected under 35 U.S.C. §§ 102(a) and 102(e) as allegedly being anticipated by Baughn et al. (WO 02/40671). The rejections are respectfully traversed.

It is respectfully submitted that a two-prong inquiry must be satisfied in order for a Section 102 rejection to stand. First, the prior art reference must contain all of the elements of the claimed invention. *See Lewmar Marine Inc. v. Barient Inc.,* 3 U.S.P.Q.2d 1766 (Fed. Cir. 1987). Second, the prior art must contain an enabling disclosure of the claimed invention. *See Chester v. Miller,* 15 U.S.P.Q.2d 1333, 1336 (Fed. Cir. 1990).

As amended herein, the pending claims require that that the isolated polypeptide comprises or consists of i), ii), or iii), and that the polypeptide additionally comprises an

extracellular domain as recited in SEQ ID NO: 22, and that the polypeptide functions as an antagonist of cytokine expression and/or secretion.

In contrast, Baughn *et al.* does not disclose the existence of an extracellular domain in the IGSFP-4 polypeptide, let alone the sequence of this domain. It does not therefore disclose an isolated polypeptide comprises or consists of i), ii), or iii), and that the polypeptide additionally comprises an extracellular domain as recited in SEQ ID NO: 22, and that the polypeptide functions as an antagonist of cytokine expression and/or secretion, and therefore fails to teach or suggest all of the elements of the pending claims. That is, Baughn *et al.* fails to teach an isolated polypeptide that comprises one of SEQ ID NOs: 16, 20 or 26 in combination with SEQ ID NO: 22, wherein the polypeptide functions as an antagonist of cytokine expression and/or secretion, and for this reason the rejection must fail.

Accordingly, as Baughn fails to teach or suggest all of the elements of claim 1, claim 1 and those claims depending therefrom, are necessarily patentable over Baughn either alone or in combination with Ruben, and the rejections must be withdrawn. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. §§ 102 and 103 are respectfully requested.

REQUEST FOR INTERVIEW

If any issue remains as an impediment to allowance, prior to issuance of any paper other than a Notice of Allowance, an interview, is respectfully requested, with the Examiner and the Examiner's supervisor, and, the Examiner is respectfully requested to contact the undersigned to arrange a mutually convenient time and manner for such an interview.

CONCLUSION

In view of the amendments, remarks and enclosures herein, the application is in condition for allowance. Reconsideration and withdrawal of the rejections of the application, and prompt issuance of a Notice of Allowance, is respectfully requested.

Respectfully submitted,
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Annex 1

Example 1: Cytokine expression modulation properties of INSP052EC-6His in LPS-induced cytokine release in mice

1.1: Introduction

The ability of INSP052EC to protect from the effects of cytokine release *in vivo* has been also tested by injecting either the recombinant protein or encapsulated, transiently transfected HEK293 cells expressing INSP052EC-6His in the model of LPS-induced TNF alpha and IL-6 release in mice.

Encapsulation of cells expressing a recombinant protein allows understanding of the possible therapeutic effects of a continuous administration of the protein *in vivo,* as shown with proteins with tumor suppressor function, for example (Visted T *et al.*, 2003, Hum Gene Ther., 14, 1429-40).

LPS (Lipopolysaccharides) are an important component of the outer membranes of gram-negative bacteria and are the best characterised example of innate recognition that leads to a robust inflammatory response by macrophages or microglia cells via its binding to CD14 and the Toll receptor 4 (Lehnardt S *et al.*, 2002, J Neurosci., 22, 2478-2486). LPS are widely used in literature to activate various cell types like macrophages, microglia and endothelial cells, in particular in relationship to liver diseases (Jirillo E *et al.*, 2002, J Endotoxin Res., 8, 319-327).

1.2: Materials & Methods

1.2.1: Encapsulation of transiently transfected HEK293 cells expressing INSP052EC-6His

1.2.1.1: Cell maintenance

Human Embryonic Kidney 293 cells expressing the Epstein-Barr virus Nuclear Antigen (HEK293-EBNA, Invitrogen) were maintained in suspension in Ex-cell VPRO serum-free medium (maintenance medium, JRH, UK) supplemented with 4mM L-Glutamine (Invitrogen) and 1ml/L Phenol-Red-solution (0.5% w/v in water, Phenol Red: Sigma, USA) in spinner flasks (Techne, UK).

1.2.1.2: Cell transfection

At the day of transfection cells were centrifuged and re-suspended in a spinner vessel (DasGip, D) in 250 mL DMEM / F12 (1:1) medium containing 1% FBS and 4ml/l ITS-X supplement (seeding medium, all Invitrogen) at a density of $1x10^6$ cells/ ml. Cells were transfected using the PEI method with a ratio of 2:1 PEI:DNA. In 100 mL seeding medium 500µg of corresponding plasmid (pDEST12.2-INSP052EC) was mixed with 1 mg PEI (Polysciences, USA) and incubated for 10 minutes at room temperature. The mixture was added to the cell suspension and incubated for 90 minutes at 37°C. After the incubation the cell suspension was centrifuged (200xg, 10 minutes at 4°C) and the cell pellet was re-suspended in 500 ml maintenance medium. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C until encapsulation.

1.2.1.3: Cell encapsulation

HEK293EBNA cells transfected with pDEST12.2-INSP052EC or not transfected (control cells) were encapsulated into Alginate-poly-L-Lysine-Alginate (APA) capsules using the Inotech research encapsulator (Inotech, CH). Cells were centrifuged (200xg 10min 4°C) and re-suspended in 2 ml washing buffer (all chemicals Inotech, CH). To this suspension a 1.5% alginate solution was slowly added to yield a final cell concentration of 2.5x10e6 cells/ml solution. The alginate-cell-suspension was taken up into a syringe (Braun Omnifit, Braun, D), which was connected to the encapsulation machine.

The encapsulation was carried out using the following parameters:

- Syringe Pump: 275 (50ml Syringe) or 456 (20ml Syringe)
- Anode voltage: 1.16kV
- Vibration frequency: 1943 Hz
- Vibration amplitude: 3

The protocol for encapsulation was the following:

- Polymerisation buffer: 10 minutes (volume 250 ml)
- Poly-L-Lysin: 10 minutes (volume 150 ml)
- Washing buffer: 1 minute (volume 150 ml)

- Washing buffer: 5 minutes (volume 150 ml)
- 0.03% Alginate: 5 minutes (volume 150 ml)
- Washing buffer: 1 minute (volume 150 ml)
- Depolymerisation buffer: 10 minutes (volume 300 ml)
- Washing buffer: 1 minute (volume 150 ml)
- Washing buffer: 5 minutes (volume 150 ml)
- Medium (Excell-V-Pro): volume 100 ml

All buffers were prepared according to the manufacturer's manual in sterile distilled water under sterile conditions. In the final step of the encapsulation, the capsules were re-suspended in 100 ml maintenance medium and transferred into a sterile spinner vessel (Dasgip, D). The capsules were incubated in a humidified atmosphere with 5% CO_2 at 37°C overnight or until injection into the animals.

1.2.2: LPS induced cytokine release model *in vivo*

The model of LPS-induced TNF alpha and IL-6 release in mice was set up according to WO98/38179. Briefly, male C57/BL6 or C3H/HeN mice (8 weeks of age; Charles River, France) were used. In general, 10 animals per experimental group are used. Mice were maintained in standard conditions under a 12-hour light-dark cycle, provided irradiated food and water ad libitum.

LPS (O111:B4 (Sigma, Switzerland), 0.3 mg/kg) was injected s.c in mice. Ninety minutes later blood was sampled and plasma TNF alpha was determined using an ELISA kit (R&D). IL-6 levels were measured after 150 minutes using a commercial available ELISA kit (R&D Duoset ref. DY206). Dexamethasone, the reference compound, was solubilized in PBS and Dexamethasone (0.1 mg/kg, s.c.) was injected 15 minutes prior LPS.

The suspension containing the microcapsules containing HEK293 cells (control cells or cells transiently expressing INSP052EC-6His) was removed from the incubator and left several minutes in the laminar flow hood to allow the capsules to sediment. The clear supernatant was removed and the concentrated capsules were taken up carefully into a

syringe. 700µl capsules were injected slowly i.p. via a 0.7 mm needle (ref 53158.01 Polylabo, CH) per mouse. LPS injection was performed at day 3 after the injection of the capsules.

1.3: Results

The potential of INSP052EC to downregulate LPS-induced TNF alpha or IL-6 release in the blood was demonstrated in both models of INSP052EC administration.

The injection of INSP052EC-6His 15 minutes prior to the LPS injection. decreases LPS-induced release of IL-6 (if INSP052EC-6His is administered at least at 0.1 mg/kg) and TNF alpha (if INSP052EC-6His is administered at least at 1 mg/kg) in a statistically significant manner, similarly to the reference compound Dexamethasone. Mice injected with the vehicle solution for injection (PBS-BSA with 0.02% glycerol) were used as negative controls (Figure 1).

Similar positive effects were observed when the HEK293 cells transiently expressing INSP052EC-6His were injected in all the tested capsule volumes (Figure 2).

Example 2: Properties of INSP052EC-6His in a model of Contact Hypersensitivity

2.1: Introduction

INSP052EC was tested on hapten induced contact hypersensitivity (CHS), a murine model of inflammatory skin disease. CHS is a T cell-mediated inflammation model of the skin that represents a well established model for similar inflammations associated to diseases such allergic contact dermatitis and psoriasis, which are dermatological problems with unmet medical needs related to excessive cytokine production (Nakae S *et al.*, 2003, Int Immunol., 15: 251-260; Gorbachev AV and Fairchild RL, 2001, Crit Rev Immunol., 21: 451-72).

2.2: Material and Methods

The hapten DNFB (2,4-dinitrofluorobenzene; Sigma Chemical Co.) was diluted in acetone / olive oil (4:1) immediately before use. Mice were sensitized with 30 µl of 0.5% DNFB solution painted to the shaved dorsal skin or left untreated. Mice were challenged

five days later, *i.e.* CHS was elicited by applying a non-irritant dose of 10 µl of 0.2% DNFB onto both sides of the right ear and the same amount of solvent alone onto the left ear. Ear thickness was monitored at day 6 using a caliper (Mitutoya).

Ear swelling was calculated as

$$((T_6\text{-}T_5) \text{ right ear}) - ((T_6\text{-}T_5) \text{ left ear})$$

where T_6 and T_5 represent values of ear thickness at day 6 and day 5, respectively, after sensitization challenge, respectively. To assure that the observed swelling was due to DNFB specific inflammation rather than non-specific irritation, a non-sensitized but challenged group of mice was included with each experiment.

Mice were treated on Day 5 with an s.c injection of INSP052EC-6His in the indicated amount, Dexamethasone (1 mg/kg), or PBS only (control group).

2.3: Results

We show that INSP052EC reduces ear swelling in significant and dose dependent manner, suggesting a decrease in leukocyte infiltration and of the consequent inflammation (Figure 3), demonstrating that INSP052EC can be useful in treating T cell-mediated inflammation of the skin, such as allergic contact dermatitis and psoriasis.

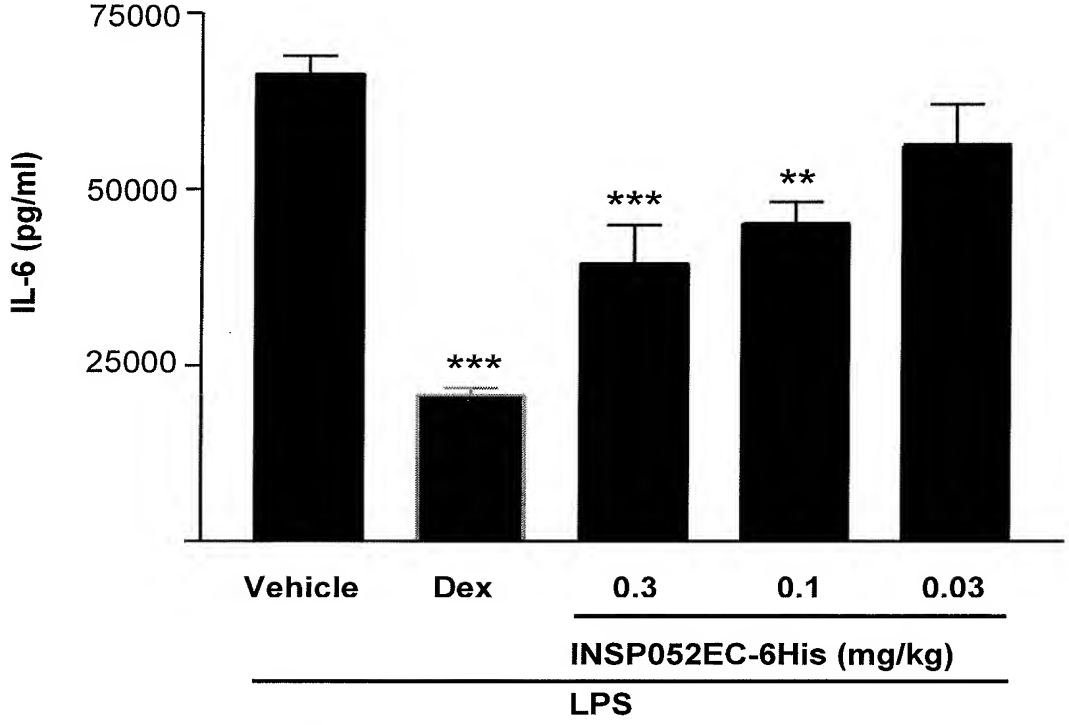
The examples clearly show that the isolated extracellular domain of INSP052 (INSP052EC) can be used (as such or as a variant or a fusion protein containing this protein sequence or the full length protein) for modulating cytokine activities, in particular as antagonist of cytokine secretion and/or expression, and may have a therapeutic role in diseases directly or indirectly related to both innate and adaptive immune responses.

The range of inhibiting activities shown by the tested INSP052EC-based molecule in different cell-based assays and animal models confirms that patho-physiological effects of cytokines resulting from their excessive or inappropriately localized production can be blocked by using this molecule. The control of cellular events associated to prolonged production of proinflammatory cytokines can be obtained by INSP052EC-based molecules, which therefore can be used for antagonizing abnormal inflammatory states associated, in particular, to autoimmune and inflammatory diseases affecting various

tissues and organs (*e.g.* liver, skin, lungs, central nervous system), providing as well a new therapeutic opportunity for oncological, neurological, cardiovascular, and infectious disorders. Additional clinical applications for INSP052EC-based molecules can be identified by using cytokine assays showing the excessive expression and / or secretion of cytokines in samples obtained by patients affected by other diseases (Wong CK and Lam CW, Adv Clin Chem. 2003, 37:1-46; Whiteside TL, Biotechniques, 2002, Oct. Suppl:4-8, 10, 12-5), then justifying the therapeutic use of a cytokine antagonist as INSP052EC-based molecules.

Figure 1

A)


75000
50000
25000
IL-6 (pg/ml)
***
***
**
Vehicle
Dex
0.3
0.1
0.03
INSP052EC-6His (mg/kg)
LPS

B)

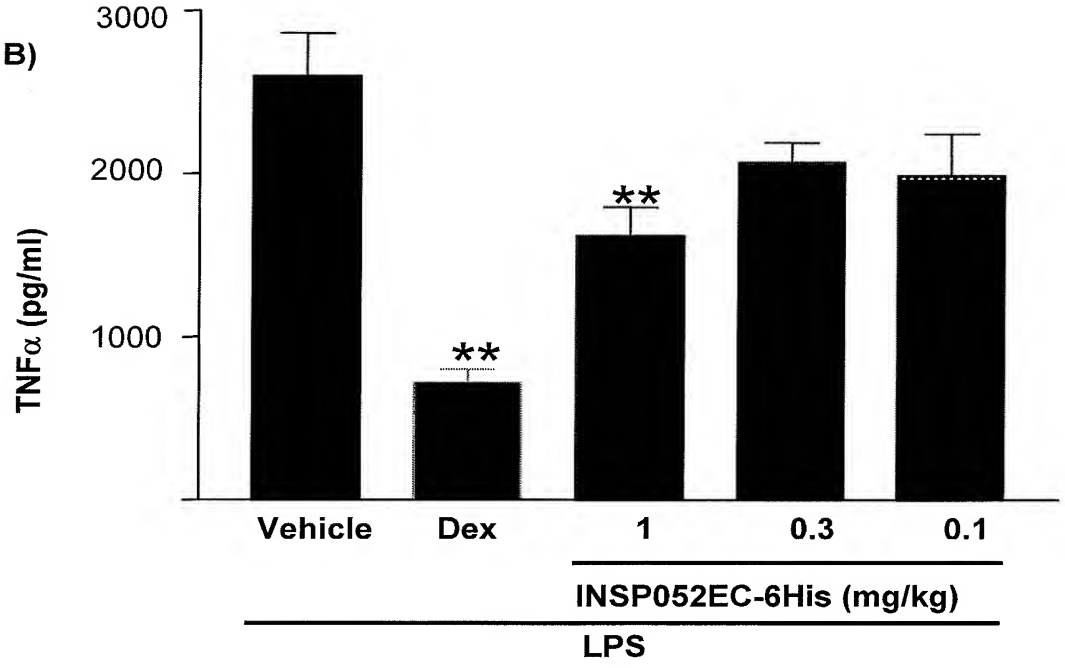

3000
2000
1000
TNFα (pg/ml)
**
**
Vehicle
Dex
1
0.3
0.1
INSP052EC-6His (mg/kg)
LPS

Figure 2

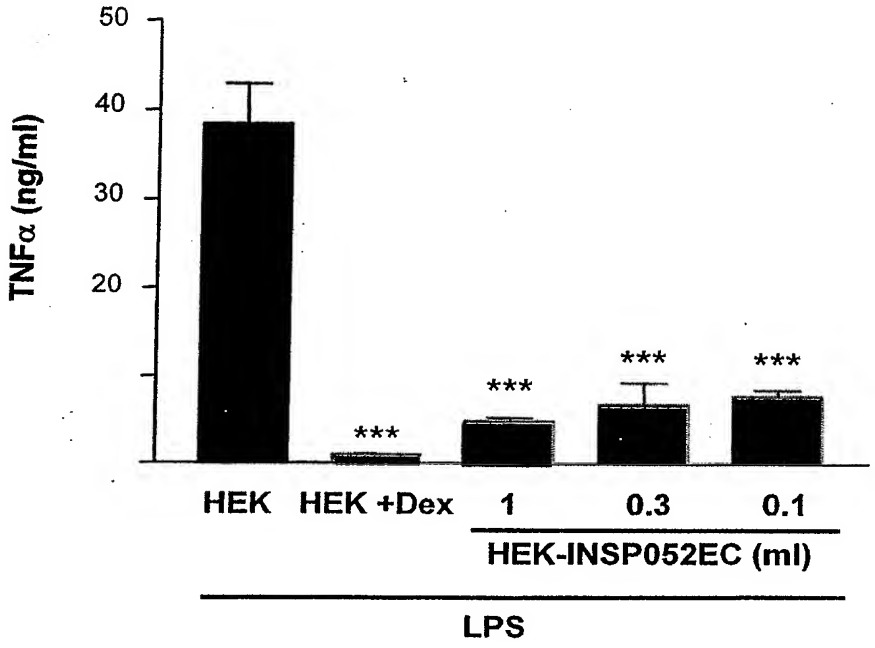
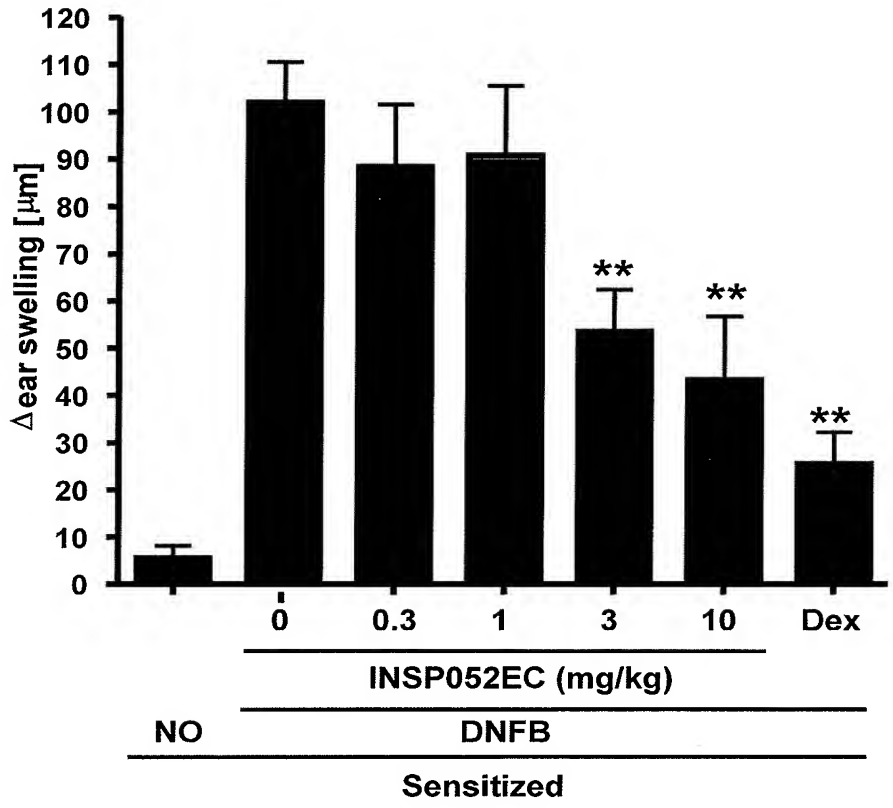


Figure 3


120
110
100
90
80
70
60
50
40
30
20
10
0
Δear swelling [μm]
**
**
**
0
0.3
1
3
10
Dex
INSP052EC (mg/kg)
NO
DNFB
Sensitized

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Cloning and characterization of hepaCAM, a novel Ig-like cell adhesion molecule suppressed in human hepatocellular carcinoma

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***Background/Aims*: Previously, we reported on gene HEPN1 that was silenced in hepatocellular carcinoma (HCC) and its capability of arresting cell growth. In this study, we identified another novel gene hepaCAM from the liver, which contains the full-length HEPN1 on its antisense strand in the 3′-noncoding region, and assessed its expression, characteristics and functions in HCC.**

***Methods*: Full-length hepaCAM cDNA was isolated by rapid amplification of cDNA ends. The gene expression was examined by semi-quantitative RT-PCR in 23 paired HCC liver specimens and 5 HCC cell lines. Transfection studies, coupled with immunocytochemistry, cellular interaction analyses, colony formation and microtetrazolium assay, were employed to elucidate the localization and functions of hepaCAM.**

***Results*: The expression of hepaCAM decreased in 20/23 of HCC samples and was undetectable in 5 HCC cell lines tested. The gene product consisting of 416 amino acids displayed the typical structure of Ig-like cell adhesion molecules. The protein was glycosylated and predominantly localized on the cytoplasmic membrane. When re-expressed in HepG2, hepaCAM accelerated cell spreading ($P<0.001$), increased cell motility ($P=0.0011$), reduced colony formation ($P=0.0022$), and inhibited cell growth ($P<0.001$).**

***Conclusions*: Gene hepaCAM, frequently silenced in HCC, encodes an Ig-like transmembrane glycoprotein and is involved in cell adhesion and growth control.**

***Keywords*: Hepatocellular carcinoma; hepaCAM; Ig-like cell adhesion molecule; Cell growth arrest; Cell–matrix interaction; HEPN1**

1. Introduction

Cell adhesion is crucial not only for the formation and maintenance of cellular architecture but also for the normal biological processes including adhesion, migration, proliferation and survival [1]. Such specialized recognition and adhesion are mediated by cell adhesion molecules (CAMs) expressed on the cell surface. Generally classified into cadherins [2,3], selectins [4], integrins [5], and immunoglobulin superfamily (IgSF) [6], these glycoproteins recognize and interact either with other cell adhesion molecules on the adjacent cell surface or with proteins deposited in the extracellular matrix. In addition to the adhesive properties of these molecules, an exciting concept that has emerged from recent cell biological research is that cell adhesion complexes are not simply static architectural entities. Rather, they are dynamic units that are critical in modulating cytoplasmic signaling cascades by capturing and integrating signals from the extracellular environment [2].

Cell organization and tissue architecture of the liver are well defined. Approximately, 80% of the adult liver consists of hepatocytes that are arranged as single-cell annastomosing plates extending from the portal region of the liver lobule towards the central vein [7]. Proper liver architecture is crucial for hepatic function [8] and is commonly disrupted in disease/injury state, including hepatitis, cirrhosis [9] and hepatocellular carcinoma [10]. Disruption of normal cell–cell adhesion in transformed cells may contribute to tumor cells' enhanced

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migration and proliferation, leading to invasion and metastasis. Although the underlying mechanism of how these phenotypes are resulted remains elusive, such disruption has been related to inactivation of cadherin, or the catenin family members, and activation of signaling pathways that prevent the assembly of adherens junctions [11].

Extensive studies have shown that the disruption of cell adhesion plays a causal role in tumor progression and metastasis [12]. Alterations of several IgSF tumor suppressors have been implicated in tumor malignancies. One such intriguing Ig-containing protein is the neural cell adhesion molecule (NCAM), a cell surface sialoglycoprotein, which is involved in neural development, signal transduction and synaptic plasticity and is downregulated during tumorigenesis [13–17]. A correlation between reduced NCAM expression and poor prognosis has been reported in several cancer types, including gastrointestinal neoplasia, colorectal cancer, and pancreatic cancer [18–20]. Another IgSF adhesion protein implicated in carcinogenesis is the carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1), an epithelial cell adhesion molecule, which is frequently downregulated in liver, colorectal and prostate cancers [21–24]. Consistently, ectopic restoration of its expression in colorectal and prostate carcinoma cells significantly suppressed their tumorigenicity in vitro and in vivo [24–26], suggesting that CEACAM1 functions as a tumor suppressor gene. Moreover, CEACAM1 is an angiogenic factor and an effector of vascular endothelial growth factor in endothelial cells; and has been implicated in cell invasion and metastasis [27,28].

In our previous study, examining genes associated with human hepatocellular carcinoma (HCC) by suppression subtractive hybridization, we identified a novel gene, HEPN1, frequently silenced in HCC [29]. Interestingly, an updated BLAST search revealed that an mRNA sequence in the database (GenBank AL834419), encoding a partial open reading frame (ORF) at the 5′ terminus, contained the entire antisense strand of HEPN1 in its 3′ noncoding region. This finding led us to isolate a new gene with a full-length cDNA approximately 3.2 kb. The gene encodes a putative Ig-like cell adhesion molecule with 416 amino acids, designated as hepaCAM. In this report, we demonstrate the expression, characteristics and functions of hepaCAM in hepatocellular carcinoma.

2. Materials and methods

2.1. Isolation of hepaCAM full-length cDNA

Rapid amplification of cDNA ends was performed with the Human Liver Marathon-Ready™ cDNA Kit (Clontech) according to the manufacturer's instructions. The gene-specific primer (GSP, 5′-GCTAGGCACTCTGCTGGATGCTAGTA-3′) designed at the 5′-end on the antisense strand of HEPN1 was used with the adapter primer 1 (provided) to amplify the full-length cDNA of hepaCAM. The cDNA was cloned and sequenced.

2.2. Liver specimens and cell lines

A total of 23 paired liver specimens and 6 normal liver tissues were surgically collected at the No. 3 Hospital of Chongqing in China through Dr Yang Xiaodong. The final diagnosis of HCC was confirmed and classified by histological examination. Five human HCC cell lines, HepG2, Hep3B, Huh7, SK-Hep1 and PLC-5, were maintained in the recommended conditions.

2.3. RT-PCR

Semi-quantitative RT-PCR reactions were performed with the OneStep RT-PCR kit (Qiagen) while real-time RT-PCR was performed with the LightCycler RNA Amplification Kit SYBR Green I (Roche). A forward primer (5′-TGTACAGCTGCATGGTGGAGA-3′) and a reverse primer (5′-TCTGGTTTCAGGCGGTCATCA-3′) were used to generate a hepaCAM fragment of 235 bp from 0.2 μg of DNase-treated total RNA. Beta-actin or GAPDH was included as control.

2.4. Plasmid construct

The open reading frame of hepaCAM was generated by PCR from the full-length cDNA with the forward primer 5′-GAAGCTT(*Hind*III)-CAAAATGGAGAGAGAAAGGGGAGCC-3′ and the reverse primer 5′-AGGATCC(*Bam*HI)-GGCCCAGGCGCTGATCTCCACC-3′. The PCR product was cloned into the *Hind*III/*Bam*HI restriction sites of pcDNA6/V5-His (Invitrogen). The construct, namely hepaCAM-V5, facilitated the expression of hepaCAM-V5 fusion protein and the detection by anti-V5 antibody.

2.5. Transfection

Transient transfections were carried out with Lipofectamine Plus (Invitrogen). Hep3B and HepG2 cells grown on coverslips were transfected with either hepaCAM-V5 or pcDNA6/V5-His (pcDNA6) vector for 48 h before immunocytochemistry. Stable transfections were performed on HepG2 cells. Transfected cells were selected in the presence of 10 μg/ml of blasticidin (Invitrogen) for 3 weeks and then cloned.

2.6. Immunocytochemistry

Cells cultured on coverslips were washed with PBS, fixed with 2% paraformaldehyde, and permeabilized with 0.2% Triton-X 100. Nonspecific sites were blocked in 10% normal goat serum (Santa Cruz). Protein expression of hepaCAM was detected using mouse anti-V5 antibody (Invitrogen) diluted at 1:200, biotin-conjugated goat anti-mouse IgG antibody (3 μg/ml), and subsequently streptavidin-fluorescein (15 μg/ml). Fluorescence was visualized by Fluorescence Microscope and Confocal Microscope LSM 510 (Carl Zeiss).

2.7. Western analysis

Total protein (50 μg) from HepG2 or liver tissue was resolved by SDS-PAGE, transblotted onto membrane, and detected by either rabbit anti-hepaCAM polyclonal antibody (generated following the procedure described in the Current Protocol) or mouse anti-V5 monoclonal antibody. The membranes were stripped and reprobed with mouse anti-GAPDH antibody (Chemicon) to assess loading quantity.

2.8. Deglycosylation

Cell lysate was deglycosylated with peptide *N*-glycosidase F (PNGase F) (New England Biolabs) according to the manufacturer's instructions. Equal amount of cell lysate without PNGase F treatment served as control. These samples were then subjected to western analysis.

2.9. Cell spreading

Cells were seeded in plates coated with 10 μg/ml-fibronectin (Santa Cruz) and incubated under standard conditions. Cell morphology was observed by microscopy (Carl Zeiss). Unspread cells were defined as round cells, while spread cells were defined as cells with extended processes [30]. The percentage of cells demonstrating spread morphology was quantified in 10 randomly selected fields (>60 cells/field).

2.10. Matrigel invasion assay

Cell migration was assessed using the transwell chambers with 8-μm pore size membranes coated with matrigel (BD Biosciences) in 24-well plates. Cells (5×10^4) were loaded into the upper volume of the chambers and allowed to migrate through the membrane for 24 h. Non-migrated cells were removed with a cotton swab, and the migrated cells were harvested by trypsinizing the lower surface of the membrane and collected into new 24-well plate. The migration activity was quantified by blind counting of the migrated cells in 10 randomly selected microscopic fields (>40 cells/field).

2.11. Wound closure assay

Cell motility was also assessed by the wound healing experiment on monolayer cells. Cells were seeded in 35-mm culture plates at high density and allowed to form monolayers overnight. Wounds were made by pipette tip on confluent cells and allowed to be healed by cell migration for 24 h. The changes in diameter (D) of each wound were measured by microscopy and computed into ratio ($D_{24\ h}/D_{initial}\times100\%$) to represent wound closure.

2.12. Colony formation

HepG2 cells transfected with either hepaCAM-V5 or vector pcDNA6 were selected in 10 μg/ml of blasticidin (Invitrogen) for 3 weeks without trypsinization while medium was refreshed every 2 days. The cell colonies formed at the end of experiment were visible, and the size and thickness of the colonies were analyzed by microscopy. The number of colonies was counted in 10 randomly selected fields.

2.13. Growth curve

The growth rate of HepG2 stable cell lines were monitored for 5 days. Cells were seeded in triplicates and cultured under standard conditions. At every 24 h, cell viability was determined by MTT assay. The growth rate of each cell line was presented as folds of increase in cell viability against the respective base line obtained on the day of seeding cells.

2.14. Bioinformatics and statistical analysis

Sequence analyses were carried out through database searches (facilitated by the NCBI, Ensembl and ExPASy). Mann–Whitney test was performed to compare two means of samples with small sample size ($n=6$). Fisher's exact test was used to assess the correlation between two parameters. Nonparametric ANOVA was performed to compare the differences between more than two means. Software InStat 3.0 (GraphPad) was employed and $P<0.05$ was considered as significant.

3. Results

3.1. Identification of hepaCAM

As illustrated in Fig. 1, the cDNA sequence AL834419 (GenBank) containing the antisense strand of HEPN1 in its 3′-noncoding region was deficient in the 5′-noncoding region. The gene specific primer (GSP) at the 5′-end of HEPN1 antisense strand and the adaptor primer (AP1) enabled us to isolate a new gene, hepaCAM, from a human normal liver cDNA library. Gene hepaCAM was mapped to human chromosome 11q24 and its genomic DNA sequence contained 7 exons ranging in sizes from 71 to 2252 bp. The full-length cDNA sequence of hepaCAM (3244 bp) has been submitted to the GenBank (AY047587).

3.2. Suppression of hepaCAM in HCC

Semi-quantitative RT-PCR revealed that hepaCAM was expressed at a similar level in all the normal liver tissues tested (Fig. 2A). To evaluate if hepaCAM expression was downregulated in HCC, we examined hepaCAM mRNA levels in 23 paired liver samples from HCC patients using a pair of hepaCAM specific primers that were not associated with the HEPN1 sequence. The results showed that hepaCAM was reduced in 87% (20/23) of HCC tissues (Fig. 2B). The expression of hepaCAM was not detectable when evaluated in 5 human HCC cell lines HepG2, Hep3B,

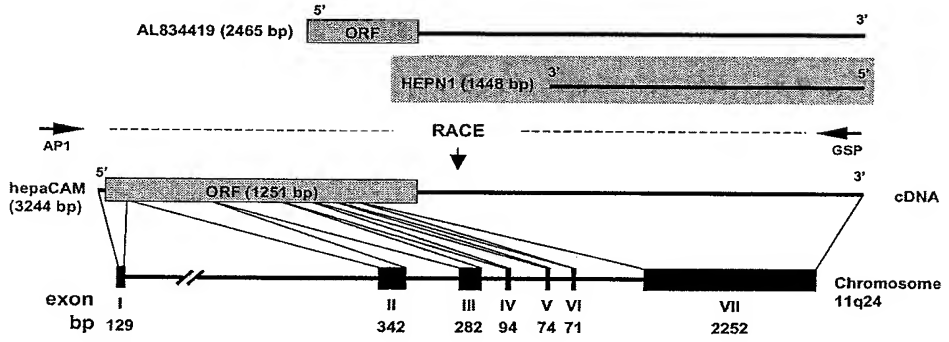

Fig. 1. Molecular cloning of hepaCAM. (A) Reconstitution scheme of hepaCAM. The full-length cDNA of hepaCAM was isolated from normal human liver cDNA library by RACE. A forward primer on the adaptor (AP1) and a gene specific primer (GSP, the reverse primer) at the 5′-end of HEPN1 antisense strand were used in the RACE reaction. The cDNA lengths of hepaCAM, HEPN1 and sequence AL834419 are given in the brackets. The indications of 5′ and 3′ correspond to the orientations of the cDNAs. The genomic DNA of hepaCAM mapped to human chromosome 11q24 contains 7 exons indicated as I–VII and accompanied by their respective length in base pairs (bp). [This figure appears in colour on the web.]

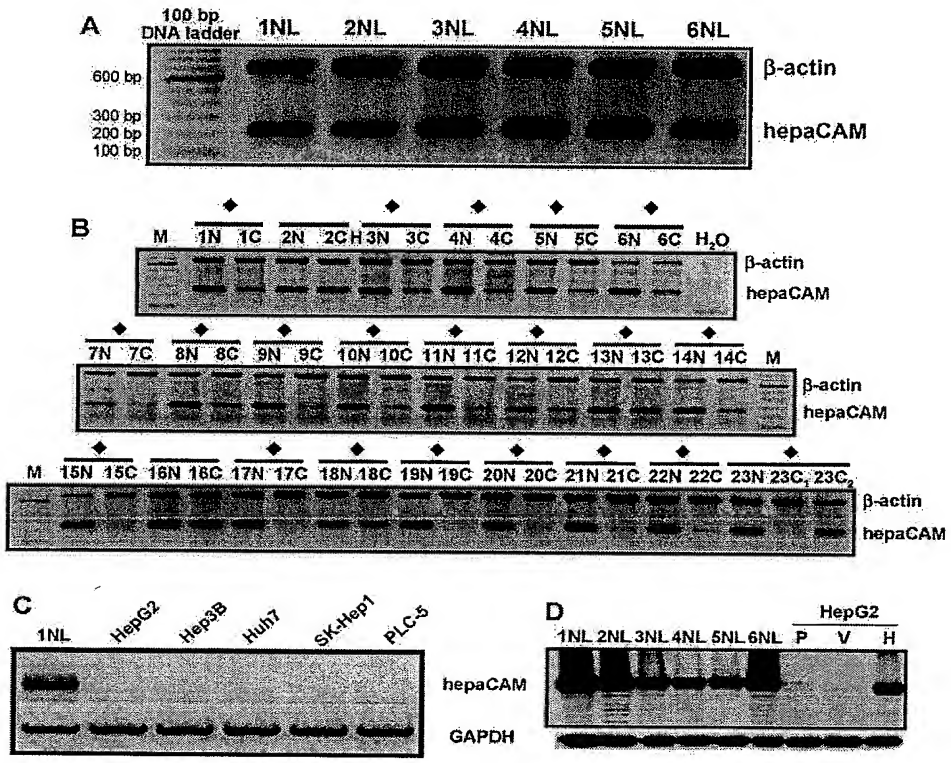

Fig. 2. Expression of hepaCAM in liver specimens and HCC cell lines. (A) Expression of hepaCAM in normal liver tissues. Semi-quantitative RT-PCR was performed to determine the mRNA expression of hepaCAM in 6 normal liver tissues obtained from 6 individuals. Two pairs of gene specific primers were included in one RT-PCR reaction to generate the fragments of genes β-actin (720 bp, as the internal control) and hepaCAM (235 bp), respectively. The samples are labeled from 1 to 6; NL, normal liver. (B) Expression of hepaCAM in 23 HCC patients. Semi-quantitative RT-PCR products were analyzed by gel electrophoresis. ◆, samples that show clear differences in hepaCAM expression; N, non-tumor liver tissue; C, HCC liver tissue; β-actin, internal control. Patient 23 had two HCC nodules (C_1 and C_2) in the liver. (C) Expression of hepaCAM in five hepatic cell lines. Semi-quantitative RT-PCR was used to amplify hepaCAM mRNA and GAPDH mRNA (internal control). The expression level of hepaCAM in 5 HCC cell lines, HepG2, Hep3B, Huh7, SK-Hep1 and PLC-5, was compared to that in the normal liver tissue 1NL. (D) Protein levels of hepaCAM in normal liver tissues and HepG2 cells. Western analysis with rabbit anti-hepaCAM polyclonal antibody revealed the endogenous hepaCAM protein levels in 6 normal liver tissues and the exogenous protein in hepaCAM-transfected HepG2 cells (H). No hepaCAM protein was detectable in both the parental (P) and the vector-transfected (V) HepG2 cells. GAPDH protein level indicates the loading quantity.

Huh7, SK-Hep1 and PLC-5 (Fig. 2C). Furthermore, western analysis with anti-hepaCAM polyclonal antibody confirmed the protein expression of hepaCAM in all the normal liver tissues, as well as in HepG2 cells transfected with hepaCAM, but neither in the parental HepG2 cells nor in the cells transfected with vector alone (Fig. 2D). These data implied the association between the loss of hepaCAM and hepatocarcinogenesis. No correlations between hepaCAM and the clinicopathologic parameters could be detected. This could be due to the high rate of hepaCAM suppression in the HCC samples tested (Table 1).

3.3. Characterization of hepaCAM protein sequence

Gene hepaCAM encoded a 46-kDa protein of 416 amino acids consisting of an extracellular region, a transmembrane segment, and a cytoplasmic tail (Fig. 3A). The extracellular region comprised a signal

Table 1
Correlation between hepaCAM suppression and the clinicopathologic parameters in 23 HCC patients

Parameters	hepaCAM suppression	Unchanged hepaCAM	Suppression rate (%)	*P*
Total number	20	3	87	
Sex				
Male	16	2	89	NS
Female	4	1	80	NS
Grade				
Well	3	0	100	NS
Moderate	12	2	86	NS
Poor	5	1	83	NS
Cirrhosis	16	3	84	NS
Hepatitis virus				
HBV	18	2	90	NS
HCV	3	0	100	NS
HBV+HCV	2	0	100	NS

Grade, histological differentiation of HCC; NS, not significant; *P* was revealed by the Fisher's exact test.

peptide and 2 Ig domains (Ig-like and C2 domains) as predicted by SignalP [31] and SMART [32], respectively. Two cysteine residues flanking the C2 domain contributed to the formation of intrachain disulfide-linked loop. Six *N*-glycosylation sites were identified in the extracellular region, which may contribute to the glycosylation of hepaCAM protein. The transmembrane segment was found to contain a prokaryotic membrane lipoprotein lipid attachment site (LLVTLVTVCAC). At the cytoplasmic tail, two potential class III PDZ domain-binding motifs were predicted. Overall, the structure of hepaCAM closely resembles Ig-like cell adhesion molecules.

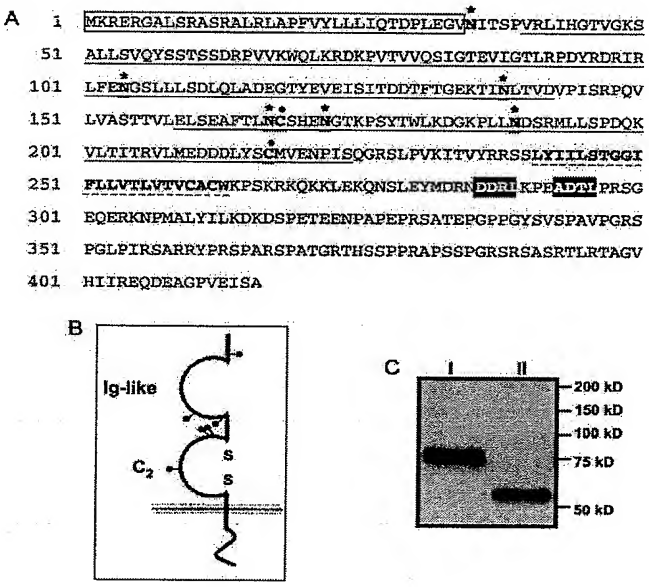

Fig. 3. The characteristics of hepaCAM protein sequence. (A) Predicted amino acid sequence of human hepaCAM. The fragment highlighted in the box is the putative signal peptide (34 amino acids). The regions underlined with solid lines are the two immunoglobulin-like (Ig-like) domains (103 and 66 amino acids, respectively) while the one underlined with dashed line is the transmembrane domain (23 amino acids). Two cysteine residues (marked underneath the '●') are identified in the second Ig-like domain, which may be needed for the formation of the disulfide bond in the domain. Six asparagines (marked underneath the '*') are in the extracellular region, which represent the potential *N*-linked glycosylation sites. The class III PDZ domain binding motifs (in the inverted shades) are present in the cytoplasmic region. (B) Illustration of the secondary structure of hepaCAM protein. hepaCAM owns the typical structure of proteins in the immunoglobulin superfamily, including an extracellular segment consisting of an *N*-terminal-proximal Ig-like domain and a membrane-proximal C_2-type Ig-domain with a disulfide bond formed between two cysteine residues (S S), a transmembrane region, and a cytoplasmic tail. The six putative *N*-linked glycosylation sites are indicated by the signs of '✓'. (C) Deglycosylation of hepaCAM. HepG2 cells transfected with hepaCAM were lysed and treated with (lane II) or without (lane I) peptide *N*-glycosidase F. The cell lysates were resolved by SDS-PAGE and subjected to western blotting with anti-V5 antibody.

3.4. Deglycosylation of hepaCAM protein

The molecular weight of the epitope-tagged hepaCAM shown by western analysis was approximately 75 kDa, larger than the predicted size (46 kDa). The six *N*-glycosylation sites identified on hepaCAM protein within the extracellular region (Fig. 3B) implied that hepaCAM protein might be glycosylated. The cleavage of *N*-linked glycans on hepaCAM by PNGase F indeed shifted the molecular weight from 75 to 60 kDa (Fig. 3C), indicating hepaCAM a glycoprotein. Noticeably, the molecular weight of the deglycosylated protein was still higher than the predicted one, suggesting the involvement of additional post-translational modifications.

3.5. Cellular localization of hepaCAM

Two hepatic cell lines Hep3B and HepG2, in which hepaCAM was undetectable (Fig. 2C), were transiently transfected with hepaCAM-V5. Immunofluorescence staining with anti-V5 antibody showed that hepaCAM was scattered in the cytoplasm, absent in the nucleus and predominantly localized on the plasma membrane of both Hep3B and HepG2 cells (Fig. 4A). Interestingly, the cellular localization of hepaCAM appeared to be cell density-dependent in HepG2 with stable transfection. In well-spread cells (Fig. 4B upper), hepaCAM was distributed in the cytoplasm and at the cell surface protrusions that were about to make cell contacts. In confluent cells (Fig. 4B lower), hepaCAM was predominantly localized on the cytoplasmic membrane, particularly in the areas of cell–cell contacts.

3.6. Evaluation of stable transfection

Two clones stably transfected with vector (V1 and V2) and 3 clones with hepaCAM-V5 (H1, H2 and H3) were screened. Western analysis showed that hepaCAM was absent in the vector clones V1 and V2, and expressed in 2 (among 3) hepaCAM clones H1 and H3 (Fig. 5A). Real-time RT-PCR showed that hepaCAM was expressed in clones H1 and H3 but not overexpressed when compared to the normal liver tissues, and was expectedly absent in clones V1, V2 and H2 (Fig. 5B). Immunofluorescence staining and confocal microscopy confirmed the establishment of cell clones (Fig. 5C). Clones V1, V2, H1 and H3 were therefore selected for the downstream functional exploration of hepaCAM.

3.7. Cell–matrix interaction and cell motility modulated by hepaCAM

As hepaCAM displayed the typical structure of cell adhesion molecules, we evaluated the adhesive properties of hepaCAM on the stable HepG2 clones through cell aggregation and spreading assays. Although hepaCAM did not clearly change cell aggregation (data not shown), it was

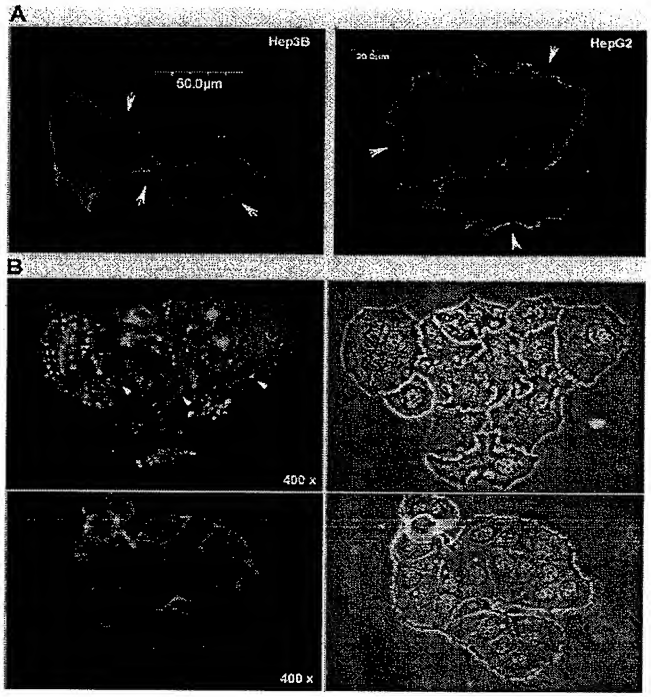

Fig. 4. Cellular localization of hepaCAM. (A) Through transient transfection and immunocytochemistry, hepaCAM protein was localized in 2 HCC cell lines. Fused with V5 in vector pcDNA6/V5-His, hepaCAM was transfected into Hep3B and HepG2 cells. Anti-V5 antibody was used for immunostaining to detect the expression and localization of hepaCAM. Confocal microscopy revealed that hepaCAM was scattered in the cytoplasm and predominantly localized on the cell membrane (arrows). (B) The localization of hepaCAM in HepG2 cells with stable transfection was cell density-dependent. Upper panel, well-spread cells with surface protrusions (arrow heads); lower panel, confluent cells with clear cell-cell contacts. [This figure appears in colour on the web.]

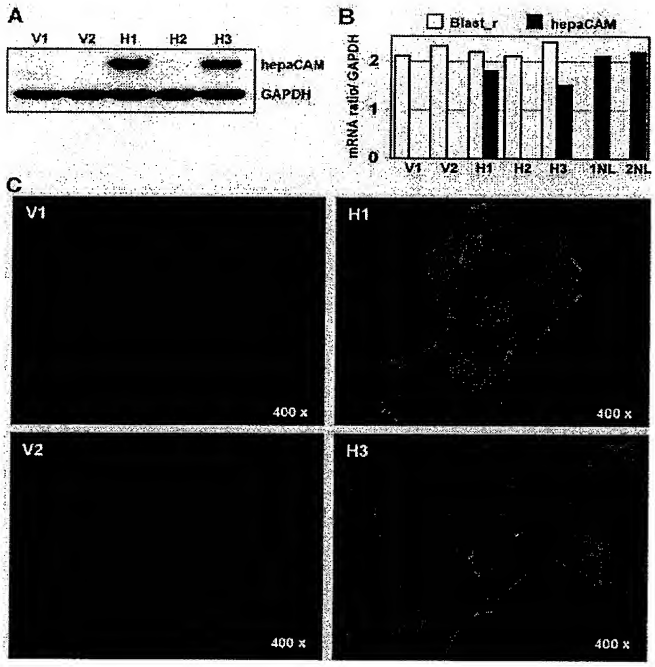

Fig. 5. Stable transfection of hepaCAM in HepG2. HepG2 cells stably transfected with vector or hepaCAM-V5 construct were cloned. (A) Western analysis. Anti-V5 antibody was used in the western analysis to evaluate the protein levels of hepaCAM in two clones transfected with vector alone (V1 and V2) and three clones transfected with hepaCAM-V5 (H1, H2, and H3). The membrane was stripped and reprobed with anti-GAPDH antibody for loading control. (B) Real-time RT-PCR analysis. The mRNA levels of blasticidin resistant gene (Blast_r) and hepaCAM were determined in all the clones and two normal liver tissues (1NL and 2NL), and converted into ratio against GAPDH mRNA levels. (C) Confocal microscopy. Immunofluorescence staining through anti-V5 antibody was used to visualize hepaCAM protein in both cells from the control clones V1 and V2 and cells from the clones expressing hepaCAM (H1 and H3). 400×, magnification. [This figure appears in colour on the web.]

capable of modulating cell–matrix adhesion significantly (Fig. 6A). About 50% and 90% of the cells from both clones H1 and H3 exhibited spread morphology at 30 min and 2 h of incubation, respectively (Fig. 6B). In contrast, the majorities of the cells from clones V1 and V2 remained round at the same time points. The number of cells showing spread morphology from clones H1 and H3 was about 5 folds higher than that from the control clones V1 and V2 ($P<0.001$). Furthermore, HepG2 cell motility was increased ($P=0.0011$) when transfected with hepaCAM as determined by matrigel invasion (Fig. 7A) and wound healing assays in culture dish (Fig. 7B). These results indicate that hepaCAM may be involved in cell and extracellular matrix interactions.

3.8. Antiproliferative effect of hepaCAM

Many Ig-like adhesion molecules, such as NCAM-1 [14], TSLC-1 [33], and OPCML [34], are known as tumor suppressors exhibiting antiproliferative effects. To examine the involvement of hepaCAM in cell growth control, colony formation was carried out while growth rate was determined in stable HepG2 clones. The results showed that the number of colonies was reduced by 10 folds in the cells transfected with hepaCAM ($P=0.0022$, Fig. 8A), and the growth rate was decreased by 14 folds ($P<0.001$, on day 5, Fig. 8B) in cells expressing hepaCAM (H1 and H3). No clear cell death was observed in the course of examining growth arrest, suggesting that hepaCAM inhibits cell growth through suppressing proliferation rather than inducing apoptosis.

4. Discussion

We have identified hepaCAM as a new Ig-like adhesion molecule. The novel protein displays the typical structure of the adhesion molecules in immunoglobulin superfamily (IgSF), including two extracellular Ig-like domains, a single transmembrane region, and a cytoplasmic tail [6].

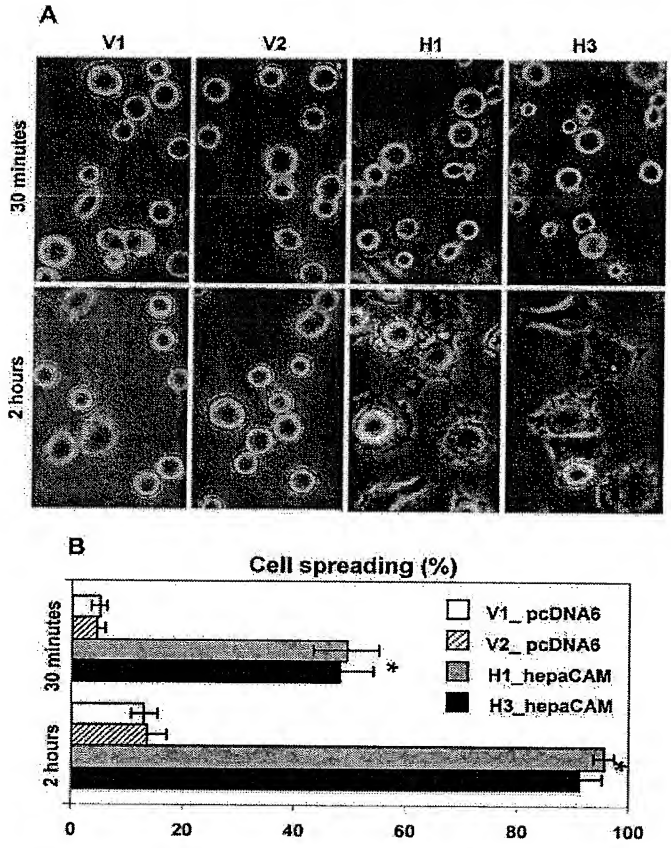

Fig. 6. Modulation of cell-matrix adhesion by hepaCAM. (A) Cell morphology. HepG2 clones transfected with vector alone (V1 and V2) and expressing hepaCAM-V5 (H1 and H3) were allowed to spread on fibronectin-coated plates for 30 min or 2 h. The microscopic photos were taken under 200×-magnification. (B) The percentage of cell spreading. At 30 min or 2 h after plating, total number of cells and cells showing spread morphology were counted in ten randomly selected fields (>60 cells per field), and the percentage of cell spreading was then computed. The data represent means±SD ($n=6$), *$P<0.001$ as assessed by ANOVA.

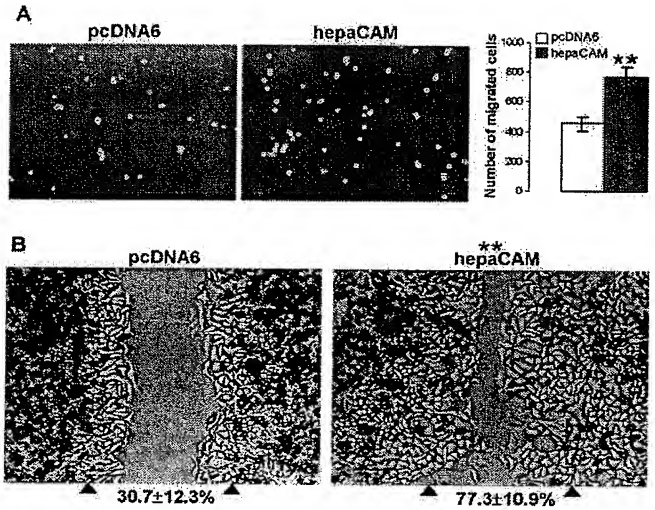

Fig. 7. Modulation of cell motility by hepaCAM. (A) Matrigel invasion assay. Cell migration was examined by using the transwell chambers with 8-μm pore size membranes coated with matrigel. HepG2 cells stably transfected with either pcDNA6 vector or hepaCAM-V5 were allowed to migrate through the membrane for 24 h. The migrated cells were harvested into new 24-well plate and viewed by microscopy (100×). The migration was quantified by blind counting of the migrated cells in 10 randomly selected fields and represented as mean±SD ($n=6$) by the bar graph. (B) In vitro wound healing assay. Wounds were made by pipette tip on confluent HepG2 cells stably transfected with either pcDNA6 vector alone (left) or hepaCAM-V5 (right) and allowed to be healed by cell migration for 24 h. The diameters of wounds were measured by microscopy (200×) at 0 h and 24 h after wounding. Arrow heads show the diameters of the initial wounds. Changes in diameter were computed into ratio (means±SD%, $n=6$) to represent wound closure. ** $P=0.0011$ as assessed by Mann–Whitney test.

The structure of hepaCAM is similar to that of adhesion molecules JAMs, CAR, and ESAM, which are known to be involved in cellular interactions. Experimentally, we have demonstrated that hepaCAM protein is glycosylated and predominantly localized on plasma membrane, particularly in the areas of cell–cell contacts when cells are confluent. Such distribution is also shown with JAMs, CAR, and ESAM. Moreover, revealed by cell spreading and motility assays, hepaCAM is capable of modulating cell–matrix interactions, further supporting hepaCAM to be an adhesion molecule.

Intriguingly, our data suggest that hepaCAM may be a tumor suppressor in human hepatocellular carcinoma. Firstly, we show that hepaCAM is expressed in all normal and non-tumorous liver tissues, but suppressed in 87% (20/23) of HCC patients and 100% (5/5) of HCC cell lines, i.e. when hepatocytes have become cancerous, indicating that loss of hepaCAM expression is associated with hepatocarcinogenesis. Secondly, the accelerated cell–matrix adhesion mediated by hepaCAM raises the possibility that loss of hepaCAM may ultimately lead to the disruption of liver tissue architecture by the loss of a tumor cell's ability to communicate with its extracellular environment. Thirdly, transfection studies revealed that hepaCAM reduced cell colony formation and inhibited cell growth in HCC cell line HepG2 through suppression of cell proliferation. The frequent loss of hepaCAM expression in HCC together with the antiproliferative effect of hepaCAM meets the most important criteria widely used to define tumor suppressor.

In addition, hepaCAM is mapped to the human chromosome 11q24. Molecular genetic and cytogenetic studies have indicated that the long arm of chromosome 11 is one of the most common targets for chromosomal aberrations during the progression of human malignancies. Tumor suppressor genes encoding cell adhesion molecules of the Ig superfamily have been identified on 11q22-qter. An Ig-like adhesion molecule TSLC1 is a tumor suppressor at 11q23 and its expression through promoter hypermethylation has been reported in the development of many human cancers such as cancers of the lung, cervix, breast and prostate [35]. However, no studies have reported

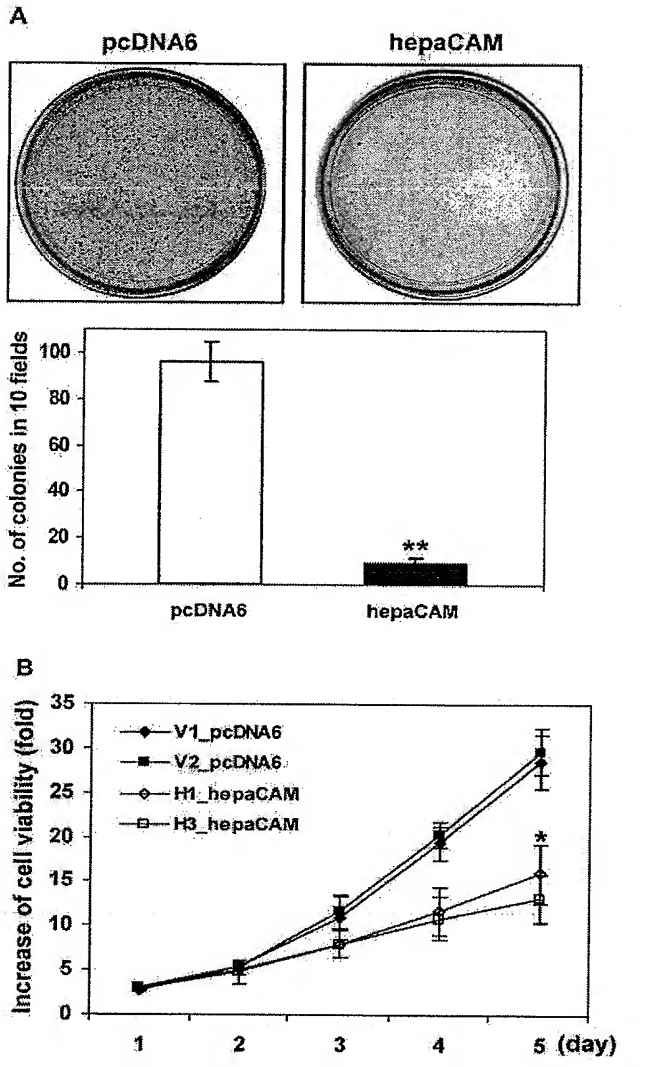

Fig. 8. Inhibition of cell growth by hepaCAM. (A) Colony formation. HepG2 cells transfected with vector (pcDNA6) or hepaCAM-V5 construct (hepaCAM) were selected with blasticidin for 3 weeks. The cell colonies formed at the end of the experiments were visible (upper panel) and the size and the thickness of the colonies were observed by microscopy. The number of colonies was counted in 10 randomly selected fields and represented by the bar graph (means±SD, $n=6$), $^{**}P=0.0022$ as assessed by Mann–Whitney test. (B) Cell growth curve. The growth rate of the cells from clones H1 and H3 (stably transfected with hepaCAM-V5) was compared to that of the cells from V1 and V2 (stably transfected with vector alone) for 5 days by microtetrazolium (MTT) assay. Data represent means±SD ($n=6$), $^{*}P<0.001$ (on day 5) as assessed by ANOVA.

the loss of heterozygosity (LOH) of chromosome 11q in HCC and the mode of hepaCAM gene silencing is yet to be understood.

In conclusion, we have identified a novel gene hepaCAM that encodes an Ig-like cell adhesion molecule. Gene hepaCAM is found frequently silenced in human hepatocellular carcinoma and the gene product is shown to be a transmembrane glycoprotein. When re-expressed in HepG2, hepaCAM is capable of mediating cell-matrix adhesion and cell motility, and exhibits antiproliferative effect. This study suggests that hepaCAM is a new Ig-like cell adhesion molecule which may play roles in cell–matrix interaction and cell growth regulation.

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Structural and Functional Analyses of a Novel Ig-like Cell Adhesion Molecule, hepaCAM, in the Human Breast Carcinoma MCF7 Cells*

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We have recently identified a novel gene, *hepaCAM*, in liver that encodes a cell adhesion molecule of the immunoglobulin superfamily. In this study, we examined the characteristics of hepaCAM protein and the relationship between its structure and function, in particular its adhesive properties. The wild-type and the cytoplasmic domain-truncated mutants of *hepaCAM* were transfected into the human breast carcinoma MCF7 cells, and the physiological and biological properties were assessed. Biochemical analyses revealed that hepaCAM is an *N*-linked glycoprotein phosphorylated in the cytoplasmic domain and that it forms homodimers through *cis*-interaction on the cell surface. The subcellular localization of hepaCAM appears density-dependent; in well spread cells, hepaCAM is distributed to cell protrusions, whereas in confluent cells, hepaCAM is predominantly accumulated at the sites of cell-cell contacts on the cell membrane. In polarized cells, hepaCAM is recruited to the lateral and basal membranes, and lacking physical interaction, hepaCAM is shown to co-localize with E-cadherin at the lateral membrane. Cell adhesion and motility assays demonstrated that hepaCAM increased cell spreading on the matrices fibronectin and matrigel, delayed cell detachment, and enhanced wound healing. Furthermore, when the cytoplasmic domain was deleted, hepaCAM mutants did not affect cell surface localization and dimer formation. Cell-matrix adhesion, however, was less significantly increased, and cell motility was almost unchanged when compared with the effect of the wild-type hepaCAM. Taken together, the cytoplasmic domain of hepaCAM is essential to its function on cell-matrix interaction and cell motility.

Cell adhesion is a dynamic process essential for the normal development and maintenance of tissues and organs in multicellular organisms. Cell-cell and cell-matrix interactions are mediated by a large and complex number of cell adhesion molecules expressed on the cell surface that interact with each other in a spatially and temporally regulated manner. According to their structural and functional features, cell adhesion molecules are generally classified into at least four major families: the cadherins, integrins, selectins, and members of the immunoglobulin superfamily (1–5). Apart from linking cells to each other or to components of the extracellular matrix, an exciting concept that has emerged from recent cell biological research is that cell adhesion molecules function also as receptors critical in modulating signal transduction (6). Such interactions are vital for the regulation of cellular adhesion, proliferation, apoptosis, migration, and differentiation.

We have recently reported the identification of a novel gene in liver, designated as *hepaCAM* (GenBank™ AY047587), which was differentially expressed in human hepatocellular carcinoma. Located on human chromosome 11q24 and spanning 7 exons, *hepaCAM* encodes a novel member of the immunoglobulin superfamily. The predicted protein of 416 amino acids displays a typical structure of Ig-like adhesion molecules, including two extracellular Ig-like domains, a transmembrane segment, and a cytoplasmic tail. In addition, when exogenously expressed in the human hepatocellular carcinoma cell line HepG2, hepaCAM accelerates cell spreading and increases cell motility (7).

The mechanism of hepaCAM in mediating cell-matrix interaction is unknown. However, transfection studies with mutant and chimeric constructs of other adhesion molecules have suggested that the structural features of adhesion molecules play important roles in mediating their physiological and biological roles. Structure and function study of E-cadherin reveals that the formation of *cis*-dimer is fundamental for cell adhesion, and inhibition of *cis*-dimer formation is correlated with the lack of cell-cell interaction (8). For CEACAM1, it has been proposed that both the first extracellular Ig domain and cytoplasmic domain are required for its adhesion function (9). Thus, defining the molecular organization of hepaCAM may help to elucidate the functional roles of hepaCAM.

In this study, we aimed to characterize the physiological and biological properties of hepaCAM and to investigate the importance of the cytoplasmic domain on hepaCAM functions in the hepaCAM-deficient MCF7 cells. We showed that hepaCAM is a phosphorylated glycoprotein that forms *cis*-homodimers on the cell surface and mediates cell-matrix interaction. In addition, the cytoplasmic domain is required for cell-matrix modulation but dispensable in subcellular localization and surface dimerization.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The complete coding sequence of *hepaCAM* and its mutants with truncated cytoplasmic domain were generated by PCR amplification. The cDNAs of *hepaCAM* residues 1–416 (wild-type), residues 1–320, or residues 1–263 were cloned into pEGFP-N2 vector (Clontech, Palo Alto, CA) or pcDNA6/V5-His vector (Invitrogen), at the HindIII/BamHI restriction sites. For polyclonal antibody generation, hepaCAM (residues 260–416) was cloned into the BglII/SalI restriction sites of the pQE40 vector (Qiagen). The sequences of the recombinant plasmids were verified by sequencing.

Cell Culture and Transfection—The MCF7 breast carcinoma cell line obtained from American Type Culture Collection (Manassas, VA) was maintained in the recommended conditions. Transfections of MCF7

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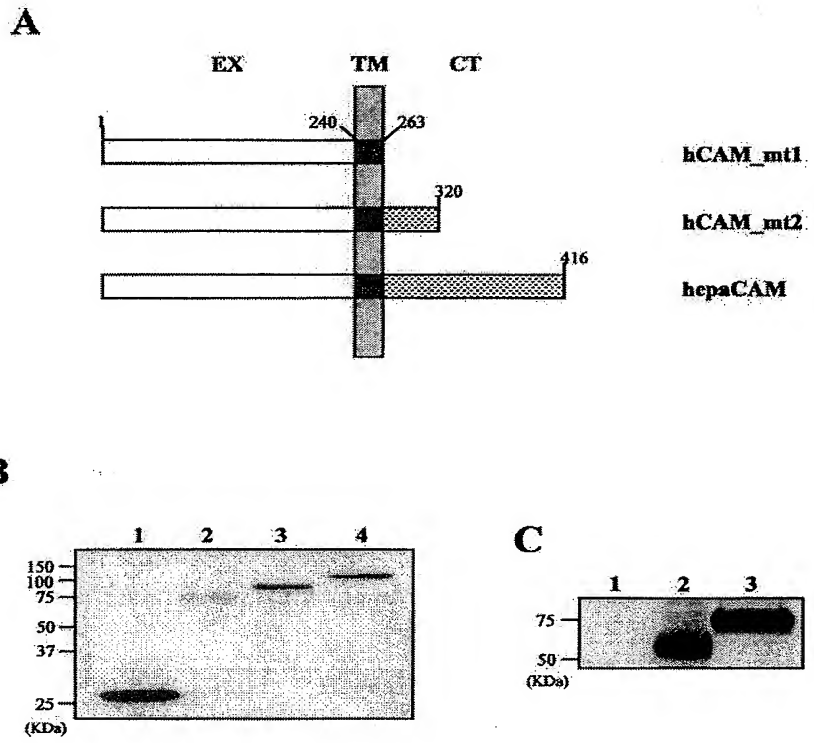


FIG. 1. **Schematic representation and expression of wild-type and cytoplasmic domain mutants of hepaCAM.** *A*, the wild-type and cytoplasmic domain mutants of *hepaCAM* were cloned into the eukaryotic expression vectors pEGFP-N2 and pCDNA6/V5-His and transfected into the breast carcinoma cell line MCF7. *EX*, extracellular domain (*white box*); *TM*, transmembrane domain (*gray box*); *CT*, cytoplasmic domain (*dotted box*). *B*, protein expression of pEGFPN2 vector and GFP-fused wild-type and mutant *hepaCAM* in MCF7 cells was detected by Western blotting using anti-GFP antibody. *Lane 1*, MCF7/pEGFPN2; *lane 2*, MCF7/hCAM_mt1-GFP; *lane 3*, MCF7/hCAM_mt2-GFP; *lane 4*, MCF7/hepaCAM-GFP. *C*, protein expression of pcDNA6/V5-His vector and V5-fused wild-type and mutant *hepaCAM* in MCF7 cells was detected by Western blotting using anti-V5 antibody. *Lane 1*, MCF7/pcDNA6; *lane 2*, MCF7/hCAM_mt1-V5; *lane 3*, MCF7/hepaCAM-V5. Positions of the molecular size markers are shown on the *left* of each panel.

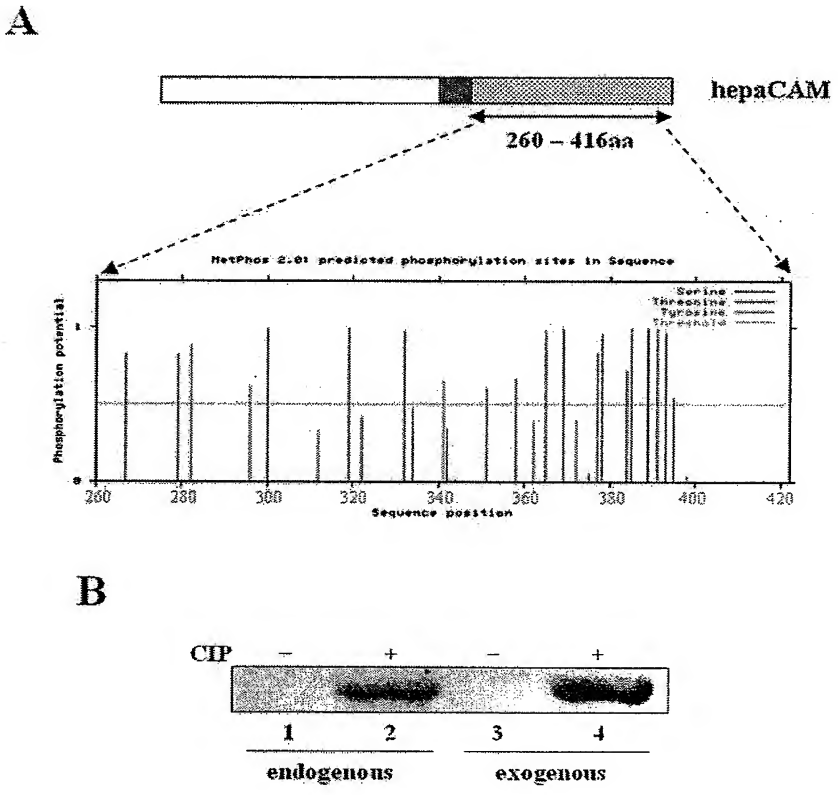


FIG. 2. **Phosphorylation of hepaCAM cytoplasmic domain.** *A*, residues 260–416 of hepaCAM was used to generate rabbit polyclonal antibody. Potential serine/threonine and tyrosine kinase phosphorylation sites in the cytoplasmic region were identified using NetPhos version 2.0 software. *aa*, amino acids. *B*, cell lysate prepared from C3A cells expressing endogenous hepaCAM (*lanes 1* and *2*) or MCF7/hepaCAM-V5 cells expressing exogenous hepaCAM (*lanes 3* and *4*) was either untreated (−) or treated (+) with calf intestinal alkaline phosphatase (*CIP*), as described under "Experimental Procedures." After dephosphorylation, hepaCAM protein was detected by Western blotting using the rabbit anti-hepaCAM polyclonal antiserum.

cells were carried out using the reagent Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected for 4 weeks, either in the presence of 600 μg/ml G418 or 10 μg/ml blasticidin, and cloned.

Western Blot Analysis—Cells were lysed in radioimmunoprecipitation assay buffer to extract the total cell lysate. Immunoprecipitation was carried out by incubating the precleared cell lysate with the appropriate mouse monoclonal antibody and horseradish peroxidase-rec-protein G (Zymed Laboratories Inc., San Francisco, CA) overnight at 4 °C. Protein was resolved by SDS-PAGE, transblotted onto membrane, and detected by either rabbit anti-hepaCAM polyclonal antiserum, mouse anti-V5 antibody (Invitrogen), mouse anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti-E-cadherin (Zymed Laboratories Inc.).

Alkaline Phosphatase Treatment—Cell lysate was incubated in de-

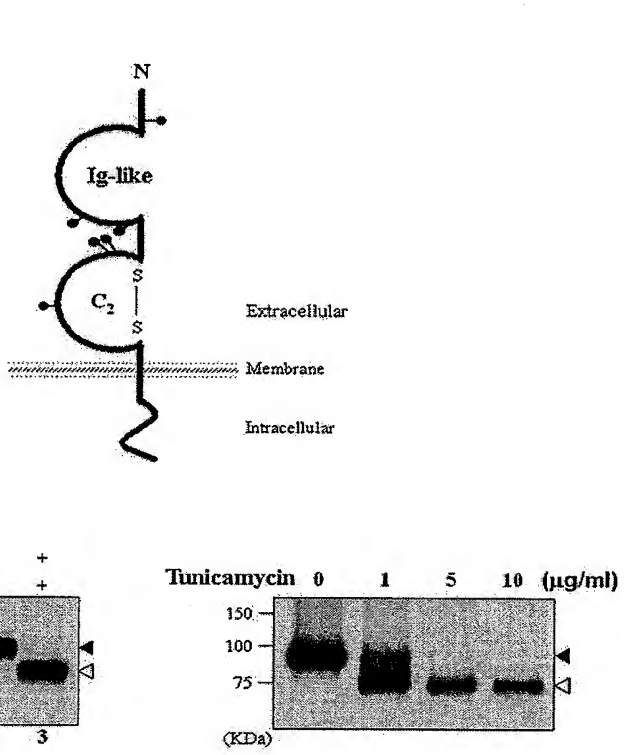


FIG. 3. ***N*-Linked glycosylation of hepaCAM-GFP.** *A*, illustration of the secondary structure of hepaCAM protein. hepaCAM owns the typical structure of proteins in the immunoglobulin superfamily, including an extracellular segment consisting of an NH_2-terminal-proximal Ig-like domain and a membrane-proximal C_2-type Ig domain with a disulfide bond formed between two cysteine residues (S—S), a transmembrane region, and a cytoplasmic tail. The six putative *N*-linked glycosylation sites are indicated by the symbol ❦. *B*, *left panel*, cell lysate was prepared from MCF7/hepaCAM-GFP (*lanes 2* and *3*) treated without (−) or with (+) *N*-glycosidase F. Untreated parental MCF7 cells (*lane 1*) were included as the control. *Right panel*, MCF7 cells transfected with hepaCAM-GFP were treated with tunicamycin at the indicated concentrations for 24 h before lysis. Protein samples were resolved by SDS-PAGE and subjected to Western blotting with anti-GFP antibody. *Solid* and *open arrowheads* indicate signals for glycosylated and deglycosylated proteins, respectively. Positions of the molecular size markers are shown on the *left*.

phosphorylation buffer for 10 min at 30 °C. Calf intestinal alkaline phosphatase (Roche Applied Science) was added and incubated for a further 15 min prior to Western analysis.

N-Linked Glycosylation Analysis—For inhibiting *N*-linked glycosylation, MCF7 cells were transiently transfected with hepaCAM-GFP and subsequently exposed to tunicamycin (Sigma) at the indicated concentrations for 24 h before lysis. For enzymatic digestion of *N*-linked oligosaccharides, the cell lysate of MCF7/hepaCAM-V5 was treated with peptide *N*-glycosidase F (New England Biolabs) according to the manufacturer's instructions. The samples were then subjected to Western analysis.

Chemical Cross-linking—A monolayer or a single suspension of cells was incubated in phosphate-buffered saline containing 3 mM BS3[1] (Pierce) or DTSSP (Pierce) at room temperature for 30 min. The reaction was quenched with the addition of 20 mM Tris-HCl, pH 7.5, for 15 min. Single cell suspension was assured by microscopic observation before and after chemical cross-linking reaction. DTSSP-cross-linked proteins were resuspended in Laemmli sample buffer without 50 mM dithiothreitol, unless indicated. Cell lysate was prepared in radioimmunoprecipitation assay buffer containing 10 mM iodoacetamide (10).

Immunocytochemistry—Cells cultured on coverslips were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. Nonspecific sites were blocked in 10% normal goat serum (Santa Cruz Biotechnology). Protein expression of V5-tagged hepaCAM was detected using mouse anti-V5 antibody, biotin-conjugated goat anti-mouse IgG antibody, and subsequently streptavidin-fluorescein. For co-localization experiments, cells were grown to confluence on 0.4-μm Transwell filters (Costar, Cambridge, MA). Protein expression of E-cadherin was detected using mouse anti-E-cadherin antibody, biotin-conjugated goat anti-mouse IgG antibody, and subsequently avidin-TRITC conjugate (Sigma). Fluorescence was visualized by fluorescence microscope (Carl Zeiss) or confocal microscope LSM 510 (Carl Zeiss) with sectioning performed at 0.5 μm.

Cell Spreading—Cells were seeded onto coverslips coated with 40 μg of matrigel basement membrane matrix (Clontech) or 10 μg/ml fibronectin (Santa Cruz Biotechnology) and incubated under standard culture conditions. Cell morphology was observed by microscopy. Unspread cells were defined as round cells, whereas spread cells were defined as cells with extended processes (11). The percentage of cells demonstrating spread morphology was quantified in 10 randomly selected fields.

Cell Detachment—A confluent monolayer of cells was detached in 1 mM EDTA in phosphate-buffered saline at 37 °C. Cell detachment was evaluated under the inverted microscope at 5 and 15 min of incubation. Concurrently, the dissociated cells were harvested and counted in 10 randomly selected fields.

Wound-healing Assay—A confluent monolayer of cells was wounded with a sterile plastic 200-μl micropipette tip. The wound was observed microscopically at 24 and 48 h. The percentage of wound filling was calculated by measuring the remaining gap space on the pictures.

Bioinformatics and Statistical Analysis—The protein sequence of hepaCAM was analyzed using the NetPhos version 2.0 and Prosite programs. Nonparametric analysis of variance was performed to compare the difference among more than two means. Software InStat version 3.0 (GraphPad) was employed, and $p < 0.01$ was considered significant.

RESULTS

Wild-type and COOH-terminal Mutants of hepaCAM—The wild-type *hepaCAM* encodes a transmembrane Ig-like adhesion molecule of 416 amino acids. To assess the importance of hepaCAM cytoplasmic domain in its physiological and biological functions, we constructed two deletion mutants of *hepaCAM*. hCAM_mt1, lacking the entire cytoplasmic tail, was constructed by truncating residues 264–416 of hepaCAM. hCAM_mt2 was constructed by deleting residues 321–416 of hepaCAM to obtain a partial cleavage of the cytoplasmic tail (Fig. 1*A*). Wild-type *hepaCAM*, hCAM_mt1, and hCAM_mt2 were fused in-frame at the NH_2-terminal of the green fluorescent protein (GFP) gene of the expression vector pEGFP-N2, and the resulting plasmids were named hepaCAM-GFP, hCAM_mt1-GFP, and hCAM_mt2-GFP, respectively. In addition, wild-type *hepaCAM* and hCAM_mt1 were inserted at the NH_2-terminal of the V5 tag of the pcDNA6/V5-His vector and designated hepaCAM-V5 and hCAM_mt1-V5, respectively. The constructs, as well as the empty vectors, were transfected into MCF7 cells, and the expressed proteins were analyzed by Western blotting using anti-GFP and anti-V5 antibodies accordingly (Fig. 1, *B* and *C*). Subsequently, MCF7 cells stably expressing pEGFP-N2 vector (MCF7/pEGFPN2), hepaCAM-GFP (MCF7/

[1] The abbreviations used are: BS3, bis(sulfosuccinimidyl) suberate; DTSSP, 3,3′-dithiobis (sulphosuccinimidyl propionate); GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate.

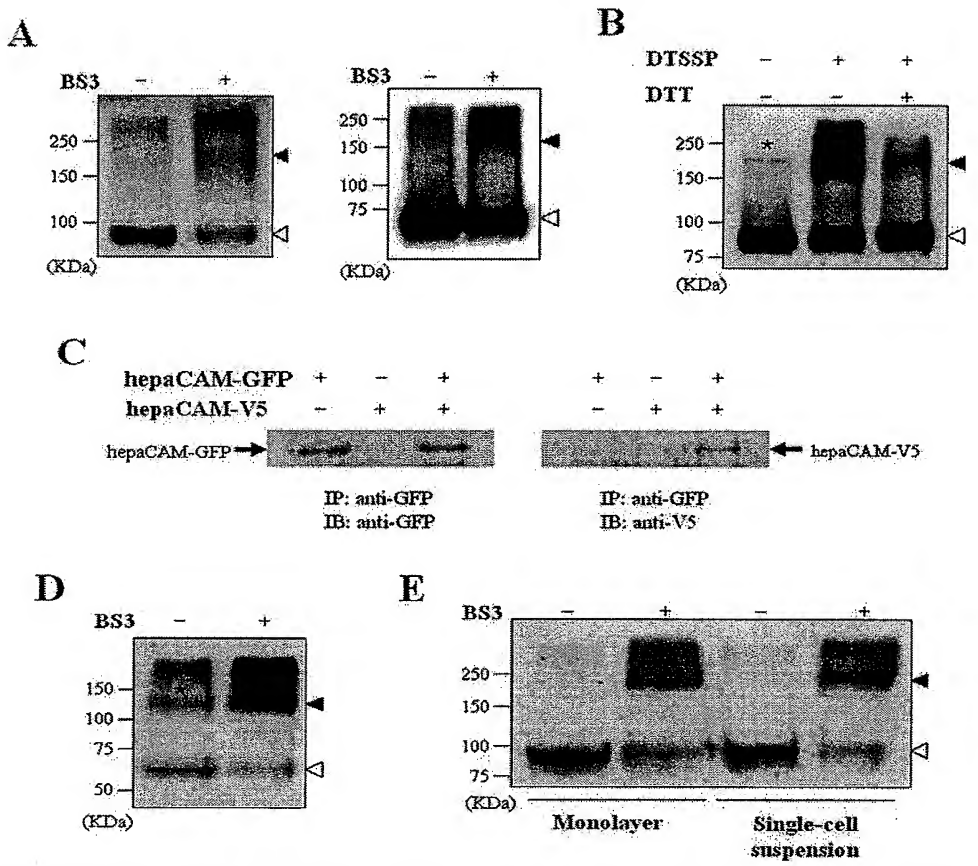


FIG. 4. **Homophilic *cis*-dimerization of hepaCAM and mutant.** *A*, cross-linking of hepaCAM-GFP (*left panel*) and hepaCAM-V5 (*right panel*) on the cell surface. A monolayer of MCF7/hepaCAM-GFP or MCF7/hepaCAM-V5 cells was untreated (−) and treated (+) with 3 mM BS3 prior to protein sample preparation in lysis buffer containing 10 mM iodoacetamide. Protein samples were subjected to Western blotting with anti-GFP antibody or anti-V5 antibody, respectively. *B*, a monolayer of MCF7/hepaCAM-GFP was untreated (−) and treated (+) with 3 mM DTSSP prior to protein sample preparation in lysis buffer containing 10 mM iodoacetamide. Protein samples were resuspended in Laemmli sample buffer in the presence (+) or absence (−) of dithiothreitol. *C*, co-immunoprecipitation of hepaCAM-GFP and hepaCAM-V5. MCF7 cells were transfected with hepaCAM-GFP, hepaCAM-V5, or both. Protein samples were prepared, immunoprecipitated with anti-GFP antibody, and subjected to Western blotting using anti-GFP antibody (*left panel*) or anti-V5 antibody (*right panel*). The signals corresponding to hepaCAM-GFP and hepaCAM-V5 molecules are marked with *arrows*. *D*, a monolayer of MCF7/hCAM_mt1-GFP cells was untreated (−) and treated (+) with 3 mM BS3 prior to protein sample preparation in lysis buffer containing 10 mM iodoacetamide. Protein samples were subjected to Western blotting with anti-GFP antibody. *E*, a monolayer and a single cell suspension of MCF7/hepaCAM-GFP were incubated in the absence (−) or presence (+) of 3 mM BS3. Protein samples were subjected to Western blotting with anti-GFP antibody. *Solid* and *open arrowheads* indicate signals for dimeric and monomeric proteins, respectively. *, dimer in un-cross-linked sample. The positions of the molecular size markers are shown on the *left* of each panel.

hepaCAM-GFP), hCAM_mt1-GFP (MCF7/hCAM_mt1-GFP), hCAM_mt2-GFP (MCF7/hCAM_mt2-GFP), pcDNA6 vector (MCF7/pcDNA6), hepaCAM-V5 (MCF7/hepaCAM-V5) and hCAM_mt1-V5 (MCF7/hCAM_mt1-V5) were generated and cloned.

Phosphorylation of the hepaCAM Cytoplasmic Domain—We generated a polyclonal antiserum that recognizes the hepaCAM cytoplasmic domain but in its dephosphorylated form. The recombinant His bacterial fusion protein used for immunization contained residues 260–416 of hepaCAM. Western analysis showed that the resulting antiserum could specifically detect the bacterial fusion protein, otherwise undetectable by the pre-immune serum. However, when the antiserum was tested on the cell lysate of MCF7/hepaCAM-V5, no specific band was observed (data not shown). We suspected that the antiserum was unable to recognize the cytoplasmic domain of hepaCAM because of the presence of post-translational modifications, *e.g.* phosphorylation. Evaluation of the region selected for antibody generation by the NetPhos version 2.0 server predicted 28 potential serine-, threonine-, or tyrosine-phosphorylated residues scattered along the cytoplasmic domain of hepaCAM protein, with 20 of them giving a potential phosphorylation >0.5 (Fig. 2*A*). To verify that the hepaCAM cytoplasmic domain is phosphorylated, we dephosphorylated cell lysates of C3A cells expressing endogenous hepaCAM and MCF7/hepaCAM-V5 cells expressing exogenous hepaCAM with calf intestinal alkaline phosphatase. The untreated cell lysates were included as controls. Indeed, calf intestinal alkaline phosphatase-treated endogenous and exogenous hepaCAM were detected by the rabbit antiserum (Fig. 2*B*), confirming that the cytoplasmic domain of hepaCAM is phosphorylated.

N-Linked Glycosylation of hepaCAM—Sequence analysis of hepaCAM predicted six *N*-linked glycosylation sites on its extracellular domain (Fig. 3*A*). To investigate whether hepaCAM was glycosylated, the MCF7/hepaCAM-GFP cell lysate was enzymatically digested with peptide *N*-glycosidase F to release putative *N*-linked oligosaccharides. An untreated sample was included as the control. The molecular mass of hepaCAM-GFP, shown by Western analysis to be ~100 kDa, was shifted to ~75 kDa after deglycosylation. Consistently, when MCF7 cells transfected with hepaCAM-GFP were treated with tunicamycin (an antibiotic that inhibits *N*-linked glycosylation) at dif-

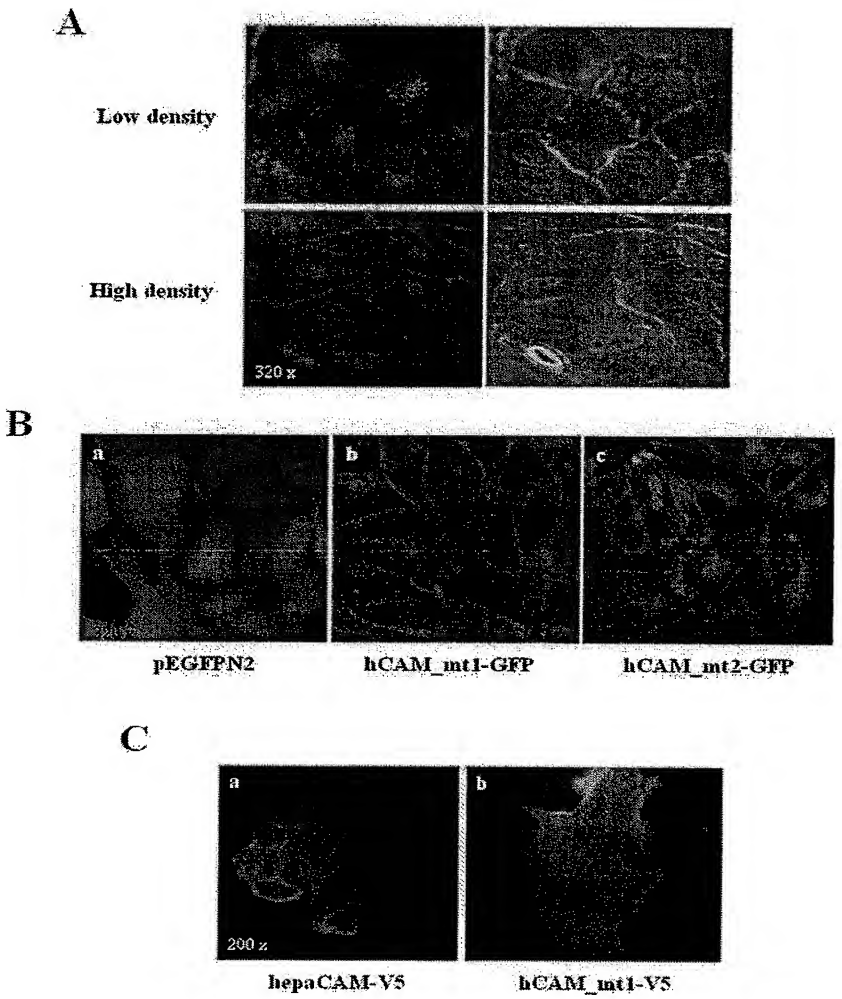


FIG. 5. **Subcellular localization of hepaCAM and mutants in MCF7 cells.** *A*, the localization of hepaCAM-GFP in MCF7 cells is cell density-dependent. MCF7/hepaCAM-GFP cells were seeded at low density and cultured for a few days. Cells at areas of low density (*top panels*) and high density (*bottom panels*) were observed under a fluorescence (*left panels*) or inverted microscope (*right panels*). Magnification is ×320. *B*, the expression of pEGFP-N2 (*a*), hCAM_mt1-GFP (*b*), and hCAM_mt2-GFP (*c*) in MCF7 cells was detected by fluorescence microscopy. Magnification is ×320. *C*, MCF7/hepaCAM-V5 (*a*) and MCF7/hCAM_mt1-V5 (*b*) cells were immunostained with anti-V5 antibody to detect localization of hepaCAM and mutant under a fluorescence microscope. Magnification is ×200.

ferent doses for 24 h, a band at ~75 kDa was also observed (Fig. 3*B*). The results verified that hepaCAM is a glycoprotein. By subtracting the molecular mass of GFP, *i.e.* 27 kDa, the deglycosylated form of hepaCAM is ~48 kDa.

Dimerization of hepaCAM and Mutant on Plasma Membrane—We evaluated the pre-existing forms of hepaCAM on cell membrane by incubating a monolayer of MCF7/hepaCAM-GFP cells with BS3, a noncleavable membrane-impermeable cross-linker. The cell lysate was prepared in the presence of iodoacetamide to inhibit the formation of nonspecific disulfide bonds (10). An untreated sample was included as the control. The samples were analyzed by Western blotting with anti-GFP. In the presence of BS3, a band of ~200 kDa appeared, which seemed to represent the dimerized form of hepaCAM-GFP, accompanied with the disappearance of the hepaCAM monomers. Similarly, treatment of MCF7/hepaCAM-V5 cells with BS3 resulted in a decrease of the ~75-kDa monomeric form of hepaCAM and an accumulation of the higher molecular weight species at ~150 kDa, although no distinct band was noted (Fig. 4*A*). It is possible that the anti-V5 antibody did not recognize the higher molecular weight species as efficiently as monomers. To examine whether hepaCAM forms a homodimer on the cell surface, MCF7/hepaCAM-GFP cells were treated with DTSSP, a reducible membrane-impermeable cross-linker. In the absence of the reducing agent dithiothreitol, a significant increase in the 200-kDa species was observed. However, when dithiothreitol was added into the sample buffer, the higher molecular mass was reduced to the monomeric form to a level closely comparable with that of the untreated cells (Fig. 4*B*). Additionally, we co-expressed hepaCAM-GFP and hepaCAM-V5 in MCF7 cells, immunoprecipitated the cell lysate with anti-GFP antibody, and immunoblotted with anti-V5 or anti-GFP. The result revealed co-immunoprecipitation of hepaCAM-GFP with hepaCAM-V5 (Fig. 4*C*), demonstrating that hepaCAM molecules dimerized through homophilic interaction. To examine whether tailless hepaCAM proteins form dimers, MCF7/hCAM_mt1-GFP cells were treated with BS3 and analyzed by Western blotting (Fig. 4*D*). The monomeric form of hCAM_mt1-GFP was diminished and replaced with its dimeric form at ~125 kDa in the BS3-treated sample. Interestingly, in the untreated sample of hepaCAM-GFP and hCAM_mt1-GFP, protein species that seemed to represent the dimeric form of the proteins were observed. This phenomenon could be due to covalent bonding between the dimers of hepaCAM-GFP or hCAM_mt1-GFP. To determine whether hepaCAM-GFP forms *cis*- or *trans*-dimers on the cell surface, both adherent monolayer and single cell suspension of MCF7/hepaCAM-GFP cells were treated with BS3 (Fig. 4*E*). The extent of dimerization was comparable in both adherent and suspension cells, indicating that hepaCAM homodimerization occurs predominantly through *cis*-interactions rather than *trans*-interactions within the plane of the membrane of individual cells.

Subcellular Localization of hepaCAM and Mutants in MCF7 Cells—We explored the subcellular distribution of wild-type hepaCAM in MCF7/hepaCAM-GFP cells at low and at high cell densities by fluorescence and inverted microscopy (Fig. 5*A*). When

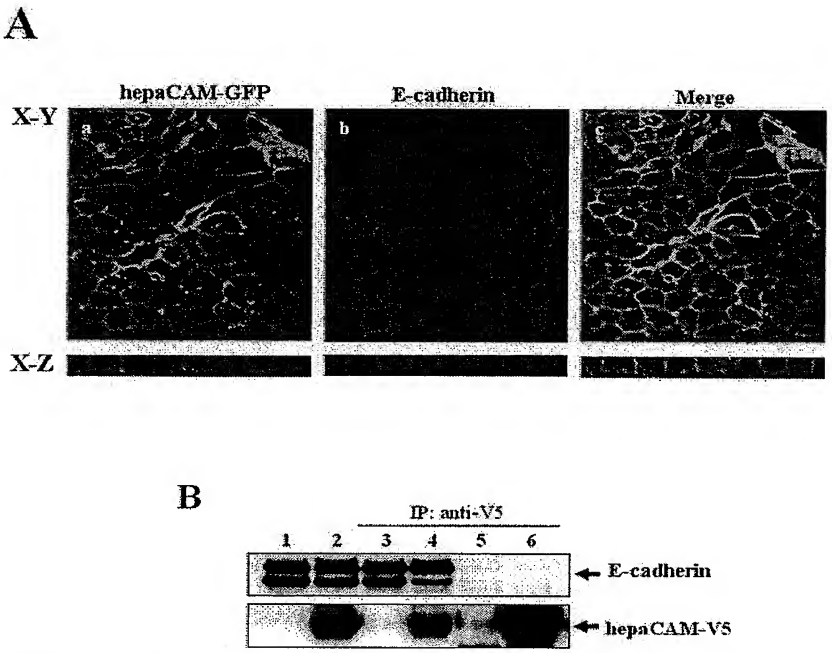


FIG. 6. **Co-localization of hepaCAM with E-cadherin.** *A*, MCF7/hepaCAM-GFP cells grown to confluence on the Transwell filter unit were fixed, permeabilized, and immunostained with anti-E-cadherin. Laser scanning confocal microscopy was performed with a filter set suitable for fluorescein and rhodamine detection. The representative sets of X-Y and X-Z sections are indicated. *a*, hepaCAM-GFP stained *green*; *b*, E-cadherin stained *red*; *c*, confocal images of the hepaCAM-GFP and E-cadherin were merged to show regions of co-localization. *B*, co-immunoprecipitation of hepaCAM-GFP and E-cadherin. Equal amounts of cell lysate prepared from MCF7/pcDNA6 or MCF7/hepaCAM-V5 cells was immunoprecipitated (*IP*) with anti-V5 antibody and subjected to Western blotting using anti-E-cadherin (*top panel*) or anti-V5 antibody (*bottom panel*). The signals corresponding to E-cadherin and hepaCAM-V5 molecules are marked with *arrowheads*. *Lane 1*, cell lysate of MCF7/pcDNA6 before IP; *lane 2*, cell lysate of MCF7/hepaCAM-V5 before IP; *lane 3*, cell lysate of MCF7/pcDNA6 after IP; *lane 4*, cell lysate of MCF7/hepaCAM-V5 after IP; *lane 5*, precipitate of MCF7/pcDNA6; *lane 6*, precipitate of MCF7/hepaCAM-V5.

cells were well spread, hepaCAM protein was localized to punctuate structures in the perinuclear membrane, cytoplasm, and at the tip of the cell surface protrusions, which were about to make contact with adjacent cell surfaces, forming zipper-like structures. Once the cells became confluent, the protein was localized at a lesser extent in the perinuclear membrane and cytoplasm and predominantly on the plasma membrane, particularly in the areas of cell-cell contacts. The results suggest that the subcellular localization of hepaCAM is cell density-dependent. We also examined the effect of hepaCAM cytoplasmic domain in its plasma membrane localization. hCAM_mt1-GFP and hCAM_mt2-GFP were both recruited to the plasma membrane of MCF7 cells (Fig. 5*B*). Similarly, MCF7/hepaCAM-V5 and MCF7/hCAM_mt1-V5 cells immunostained with anti-V5 showed that hepaCAM and its mutant were predominantly expressed on cell membranes (Fig. 5*C*). The results indicate that the cytoplasmic domain is dispensable for membrane localization.

Co-localization of hepaCAM with E-cadherin—The distribution of hepaCAM was further examined in confluent polarized MCF7/hepaCAM-GFP cells by confocal laser scanning microscopy (Fig. 6*A*). The cells were also stained for E-cadherin, which localizes in the lateral cell surface, to compare its localization with that of hepaCAM. In the X-Y sections, hepaCAM-GFP was distributed to honeycomb-like structures at cell-cell boundaries, which significantly co-localized with E-cadherin. In the X-Z vertical cross-section, the distribution of E-cadherin along the entire lateral cell surface coincided with hepaCAM-GFP. Moreover, hepaCAM was detected at the basal membrane that was in contact with the Transwell membrane. Because hepaCAM and E-cadherin appeared to co-localize, we investigated whether there were any physical interactions between them by co-immunoprecipitation (Fig. 6*B*). Cell lysate prepared from MCF7/hepaCAM-V5 was precipitated with the anti-V5 antibody and subjected to Western blotting using the anti-E-cadherin or anti-V5 antibodies. MCF7/pcDNA6 cell lysate was included in the experiment as the control. No co-immunoprecipitation was observed, suggesting that E-cadherin and hepaCAM do not physically interact.

Cell-Matrix Interaction by hepaCAM and Mutant—We evaluated the adhesive properties of V5-tagged *hepaCAM* and mutant constructs on the MCF7 cells through cell aggregation, cell adhesion, and detachment assays. No clear change in cell aggregation was observed among MCF7/pcDNA6, MCF7/hCAM_mt1-V5, and MCF7/hepaCAM-V5 cells (data not shown), but hepaCAM was capable of modulating cell-matrix adhesion significantly. Fig. 7 shows that ~60 and 79% of the MCF7/hepaCAM-V5 cells exhibited spread morphology on fibronectin at 30 min and 2 h of incubation, respectively, in contrast to 40.8 and 58.2% of the MCF7/hCAM_mt1-V5 cells and 7.3 and 18% of MCF7/pcDNA6 cells ($p < 0.001$). Similarly on matrigel, MCF7/hepaCAM-V5 cells showed the fastest spreading, followed by MCF7/hCAM_mt1-V5 cells, and then MCF7/pcDNA6 cells ($p < 0.001$). In the cell detachment assay (Fig. 8), MCF7/hepaCAM-V5 cells detached 18.9 and 21.6 times slower than MCF7/pcDNA6 cells at 5 and 15 min, respectively. MCF7/hCAM_mt1-V5 cells, on the other hand, detached ~4 and 2.2 times slower than MCF7/pcDNA6 cells at time points 5 min and 15 min ($p < 0.001$). The results showed that, in addition to its extracellular and transmembrane domains, hepaCAM needs its cytoplasmic domain to mediate strong cell-matrix adhesion.

Cell Motility by hepaCAM and Mutant—Cell motility of hepaCAM and mutant was assessed by matrigel invasion and wound-healing assays. Barely any MCF7 cells expressing pcDNA6, hCAM_mt1, and hepaCAM migrated through the 8-μm Transwell membrane (data not shown). This observation could be explained by the poorly invasive nature of MCF7 cells.

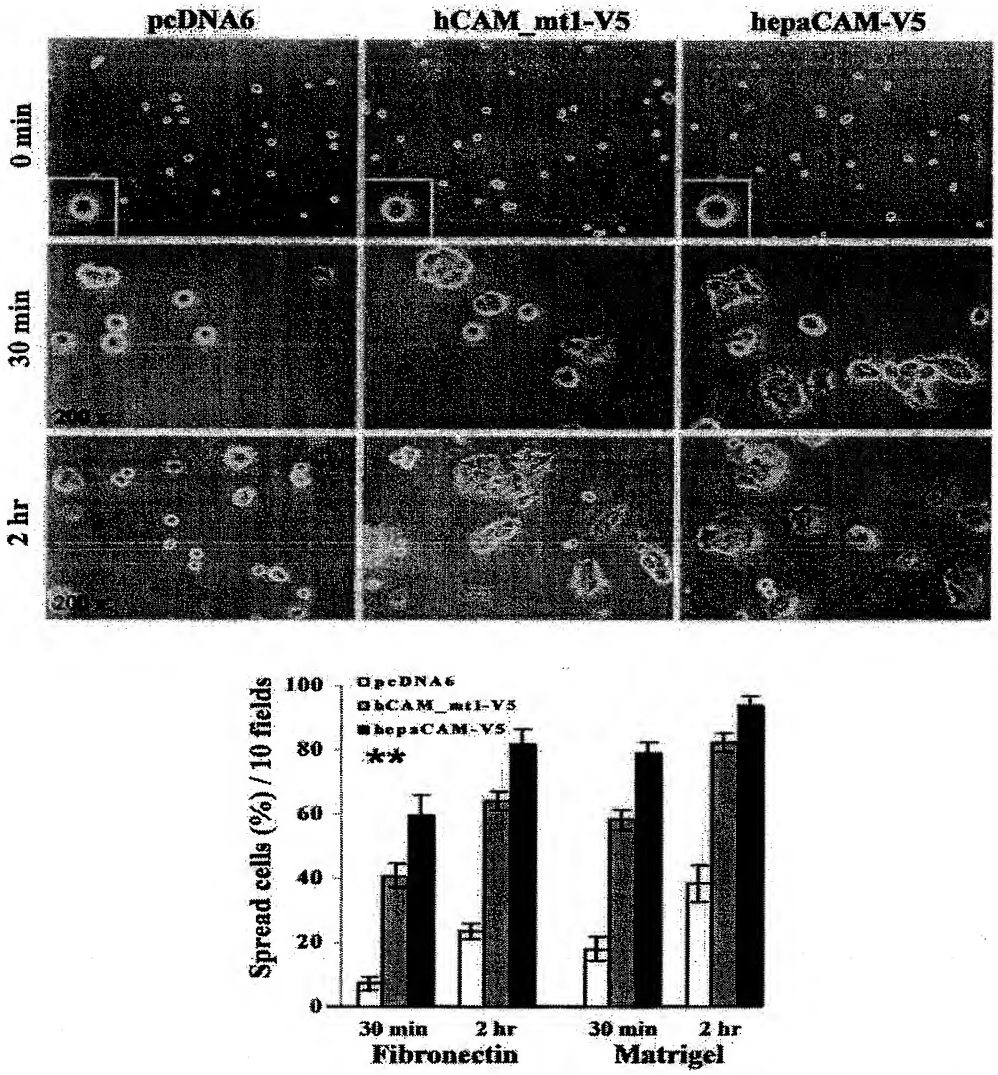


FIG. 7. **Cell spreading assay.** MCF7/pcDNA6 (*left panels*), MCF7/hCAM_mt1-V5 (*middle panels*) and MCF7/hepaCAM-V5 (*right panels*) cells were allowed to spread on matrigel-coated coverslips for 30 min or 2 h. *Insets*, cell morphology before spreading. The microscopic photos were taken under ×200 magnification. Shown is the percentage of spread cells on fibronectin and matrigel. At 30 min or 2 h after plating, the total number of cells showing spread morphology were counted in ten randomly selected fields, and the percentage of cell spreading was then computed. The data represent means ± S.D. ($n = 6$); **, $p < 0.001$ as assessed by analysis of variance.

Moreover, MCF7/hepaCAM cells were enlarged, therefore retarding migration. However, in the wound-healing assay (Fig. 9), we demonstrated that, after 24 h of incubation, MCF7/hepaCAM-V5 cells filled 59.3% of the scratched area ($p < 0.01$), compared with 36.3% by MCF7/hCAM_mt1-V5 cells ($p > 0.05$) and 33.1% by MCF7/pcDNA6 cells. After 48 h, MCF7/hepaCAM-V5 cells closed 83.7% of the wound ($p < 0.01$), compared with 55.2% by MCF7/hCAM_mt1-V5 cells ($p > 0.05$) and 49.5% by MCF7/pcDNA6 cells. Hence, the cytoplasmic domain is important for cell motility modulated by hepaCAM.

DISCUSSION

In our previous work, we identified a novel Ig-like molecule, hepaCAM, which exhibits typical structural characteristics of adhesion molecules of the immunoglobulin superfamily (7). In this study, we demonstrated physiological and biological characteristics of hepaCAM and the relationship between its structure and function, particularly with respect to the cytoplasmic domain.

Sequence analysis revealed that the cytoplasmic domain of hepaCAM contains a proline-rich region that provides putative binding sites for SH3 domains and potential phosphorylation sites of serine/threonine and tyrosine kinases. Experimentally, we showed that the cytoplasmic domain is phosphorylated, suggesting an important role of the hepaCAM cytoplasmic domain in signaling cascades controlling cellular adhesion, motility, morphology, and all processes depending on the cytoskeleton. To evaluate the significance of the cytoplasmic domain, we transfected wild-type and cytoplasmic domain-truncated constructs of *hepaCAM* into MCF7 cells and analyzed their effects on hepaCAM functions.

Biochemical analysis revealed that hepaCAM is a glycosylated protein and forms a *cis*-homodimer on the cell surface. Deletion of the cytoplasmic domain did not interfere with dimer formation, suggesting that dimerization may be stabilized by the extracellular and/or transmembrane domains but not the cytoplasmic domain. Notably, chemical cross-linking of hepaCAM or its mutated protein both showed the presence of high molecular weight proteins, indicating that hepaCAM may form large complexes with other endogenously expressed cellular proteins through its extracellular and/or transmembrane do-

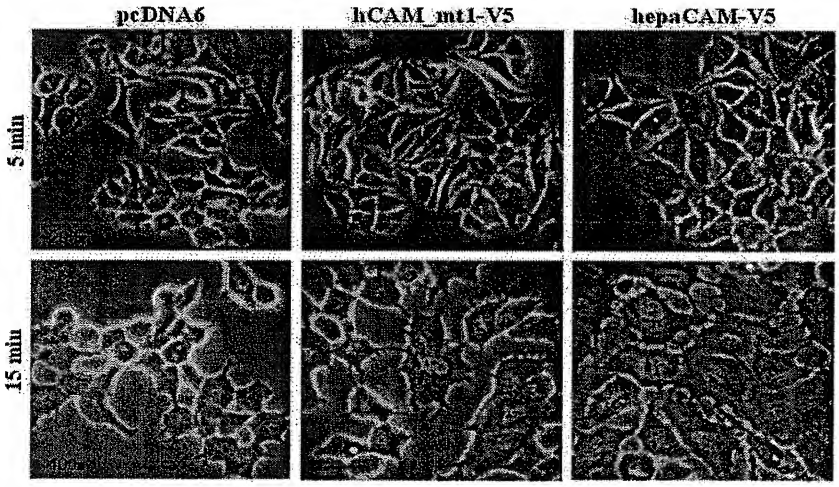
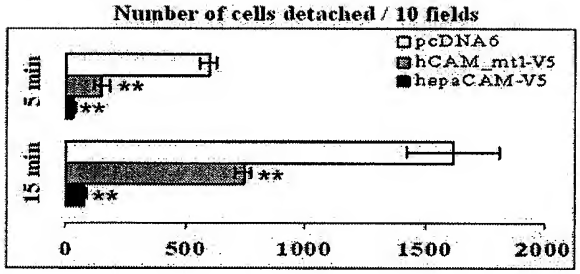
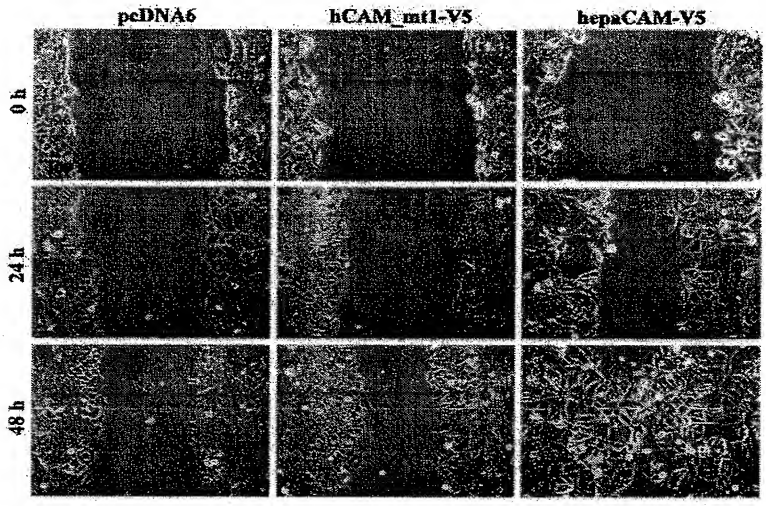


FIG. 8. **Cell detachment assay.** MCF7/pcDNA6 (*left panels*), MCF7/hCAM_mt1-V5 (*middle panels*), and MCF7/hepaCAM-V5 (*right panels*) cells were detached in 1 mM EDTA for 5 min or 15 min. The microscopic photos were taken under ×200 and ×400 magnifications. At 5 min or 15 min after incubation, the total number of detached cells was counted in ten randomly selected fields, and the percentage of cell detachment was then computed. The data represent means ± S.D. ($n = 6$), **, $p < 0.001$ as assessed by analysis of variance.





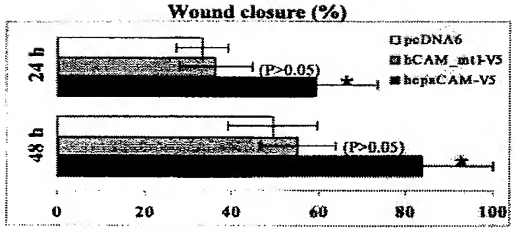


FIG. 9. **Wound-healing assay.** Wounds were made by pipette tip on confluent MCF7/pcDNA6 (*left panels*), MCF7/hCAM_mt1-V5 (*middle panels*), and MCF7/hepaCAM-V5 (*right panels*) cells and allowed to heal for 24 and 48 h. The microscopic photos were taken under ×100 magnification. The diameters of wounds were measured on the microscopic photos at 0, 24, and 48 h after wounding. Changes in wound diameter were computed into percentage (means ± S.D.%, $n = 6$) to represent wound closure. *, $p < 0.01$ as assessed by analysis of variance.

mains. Alternatively, it may represent higher order homo-oligomers of hepaCAM or its mutant. It is interesting to observe the seemingly dimeric form of hepaCAM and its mutant in their respective un-cross-linked samples. Although the mechanism resulting in such interaction is unknown to us, Hunter *et al.* (5) and others (12) have observed a similar phenomenon in C-CAM and raise the possibility that C-CAM dimers become covalently linked, perhaps through the action of transglutaminase, an enzyme which catalyzes the formation of γ-glutamyl-ε-lysine bonds in a restricted number of cellular proteins.

Subcellular localization of hepaCAM in nonpolarized MCF7 cells showed that hepaCAM molecules were recruited to the cytoplasmic membranes at sites of cell-cell attachment. In polarized cells, hepaCAM was preferentially expressed in the lateral and basal membranes. Co-localization analysis demonstrated that hepaCAM co-localized laterally with E-cadherin, but no physical interaction between the two molecules was detected. We also showed that partial truncation and complete deletion of the cytoplasmic domain did not alter the plasma membrane localization. It has been reported that the CEACAM1 cytoplasmic domain regulates its lateral localization. Differing in their cytoplasmic domains, isoform CEACAM1-S distribution is exclusively apical, whereas isoform CEACAM1-L occurs both in apical and lateral cell surfaces (13). However, whether the loss of cytoplasmic domain affects the lateral and basal localization of hepaCAM remains to be determined.

Functionally, hepaCAM is capable of modulating cell-matrix interaction. Cell adhesion to the substratum plays a crucial role in cell migration, which is a key aspect of many normal and abnormal biological processes, including embryonic development, immunity, wound healing, and metastasis of tumor cells (14, 15). The distribution of hepaCAM on the basal membrane of cells, in addition to the spread morphology of MCF7/hepaCAM-V5 cells, hinted at possible *trans*-interaction between hepaCAM and the substrate. Evidently, cell spreading, cell detachment, and wound-healing assays revealed increased cell-substrate affinity and cell motility mediated by hepaCAM. Deletion of the cytoplasmic domain reduced, but did not completely abrogate, cell-matrix adhesion mediated by the wild-type hepaCAM, implicating that, to a considerable extent, the extracellular and transmembrane domains are able to initiate adhesion. However, the rate of wound healing of cells expressing mutant hepaCAM was close to the level of the control cells, indicating that the cytoplasmic domain is essential for mediating wound recovery. The data implies that cell-matrix adhesion and cell motility are controlled separately, and phosphorylation of the cytoplasmic domain may play a pivotal role in the regulation. Indeed, phosphorylation of CD44 was shown to regulate melanoma cell and fibroblast migration on, but not attachment to, a hyaluronan substratum (16). Additionally, it has been proposed for the cadherins (8, 17, 18) and for CEA (19) that *cis*-dimerization will lead to strengthened cell adhesion, and *cis*-homodimer formation of ICAM-1 enhances its binding to a leukocyte β2-integrin (20). However, the functional significance of hepaCAM post-translational modification and dimerization in regulating cell-matrix interaction is still under investigation.

In conclusion, we have shown that hepaCAM is a phosphorylated glycoprotein, forms *cis*-homodimers on the cell surface, and modulates cell-matrix interaction. The cytoplasmic domain, although unessential for cell surface localization and dimerization, is required to maintain a complete functional form of hepaCAM as a modulator of cell-matrix interaction.

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